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Current assays for HIV-1 diagnostics and antiretroviral therapy monitoring: Challenges and possibilities

Keywords: HIV-1 diagnostic assays, viral load measurement, drug resistance monitoring, tropism determination, ultra-deep sequencing

Summary

In 2011, there were over 34 million people living with HIV infections, causing a heavy burden to public health sectors. HIV infection is a life-long threat, which cannot be prevented by vaccination and cured by antiretroviral drugs. The infected patients rely on daily antiretroviral therapy to suppress HIV viral replication. Hence, it is important to diagnose HIV infections as early as possible, and to monitor the efficacy of antiretroviral therapy every 3-6 months. Different immunoassays detecting HIV antigens and antibodies have been modified to give better sensitivity and more rapid diagnosis. Several clinical and virological parameters, including CD4+ cell counts, viral load and drug resistance mutations, are also used for treatment monitoring. Many molecular assay optimizations are now being imposed to improve patient care. This review would try to focus on the most updated HIV diagnostic assays, as well as discussing if there will be upcoming possibilities with other advance technologies.
Introduction

Nearly three decades ago, the human immunodeficiency virus (HIV) was identified to be the causative agent of the acquired immune deficiency syndrome (AIDS). [1] AIDS progression is associated with a significant decrease in CD4+ cells, causing failure in the immune systems. Based on the World Health Organization statistical data, there were over 34 million people living with HIV infections around the globe till 2011. [201] Great effort has been put into understanding the functions of different viral proteins and the viral pathogenesis inside lymphocytes. The research findings allow scientists to discover HIV antigens and antibodies for detection, antiretroviral drugs for viral inhibition, and vaccines for infection prevention and transmission.

To maximize the efficacy of patient care in HIV-infected clinics, HIV detection, viral load measurement and antiretroviral drug resistance monitoring are crucial and can be achieved by a wide range of laboratory tests. Initially, p24 viral proteins were quantified by an enzyme immunosorbent assay test. However, the amount of antigen was at limited level during the stage of acute infection. The assay sensitivity and specificity can be enhanced by the combination use of antibodies Immunoglobulin G and Immunoglobulin M test. [2] Antibodies are readily detected after seroconversion, making them the major targets in enzyme immune assays. Western blot which also detects HIV antibodies, on the other hand, is used as a
confirmation diagnostic test globally. The newly developed nucleic-acid based assays have shortened the window period from 4 weeks to 2 weeks. [3] However, the molecular testing is expensive and requires specific diagnostic machines, which is not suitable for the use in remote settings.

Zidovudine was the first nucleoside reverse transcriptase inhibitor (NRTI) approved by the Food and Drug Administration (FDA) for HIV treatment since 1987. After a few years of Zidovudine mono-therapy regimen, cases of drug resistance cases were reported. With protease inhibitors (PI) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) sequentially introduced into the market, the idea of highly active antiretroviral therapy was brought into the HIV clinics in the mid-1990s. [4] Nowadays, 3 more antiretroviral classes (fusion inhibitors, CCR5 antagonists and integrase inhibitors) are on the prescription list, covering over 25 single- or multi-class combinations of antiretroviral drugs.

Under antiretroviral drug suppression, the probability of escape mutation occurrence increases due to the fact that HIV uses error-prone reverse transcriptase for viral replication. Consequently, a series of genotypic and phenotypic assays are implemented to deduce drug susceptibility prior to and during the treatment. Besides, two other clinical parameters, CD4+ and viral load, are monitored to ensure high treatment efficacy. The CD4+ cell count is treated as a surrogate marker for observing the strength of the immune system, while the number of
viral copies is used as a prognostic marker for checking viral activity. The effectiveness of HIV RNA quantitative and qualitative assays have been improved dramatically with molecular assays. In particular, the latest technology of ultra-deep sequencing further increases the sensitivity of qualitative assays by sequencing individual amplicons. Determination of the host genetic polymorphisms has become an extra assessment for antiretroviral drug prescription due to several adverse effects and metabolic interactions.

HIV is mainly characterized into HIV-1 group M, N, O and HIV-2. The global pandemic is caused by HIV-1 group M strains while group N and O are very rare. Base on phylogenetic analysis, group M strains are further categorized into 11 subtypes, 58 circulating recombinant forms and many unique recombinant forms. [6, 7] HIV-1 subtype B and C infections are accounted for over 50% of infections worldwide. HIV-2 infections are restricted in the region of Western Africa and thus limited diagnostic development was done. [6, 8] In this review, we will focus on HIV-1 and its current diagnostic assays that are newly utilized to facilitate better detection, shorter turnaround time, and easier to manipulate for diagnosis and antiretroviral therapy (ART) monitoring.

**HIV-1 detection**

HIV-1 can be transmitted vertically by sexual contact, perinatally from mother to child, and
through contaminated blood products and needles. Certain groups of people are at high risk, including intravenous drug users, blood products recipients, healthcare workers, sexual workers and the ones who have unprotected sex and multiple partners. HIV-1 treatment is permanent and expensive. It is therefore important to detect HIV-1 in blood samples and individuals as early as possible so as to eliminate any possible infection spread.

HIV-1 detection is based on the recognition of viral antigen (p24 antigen test), antibodies (enzyme-linked immunosorbent assay, ELISA), viral proteins (western blot, WB) and nucleic acids (nucleic-acid amplification test, NAAT). During acute infection and before seroconversion, the level of antibodies is very low and only a small amount of detectable antigen is present. HIV-1 detection is usually less accurate within this one-month window period. Hence, shortening the turnaround time is always the major hurdle in upgrading the HIV-1 diagnostic assay. Apart from HIV-1 diagnosis, researchers are interested in identifying recent infection and the prevalence of infection over time. The amount of antibodies will keep rising after seroconversion for about 4 months. Using the detuned assays or sensitive/less-sensitive assays, researchers are able distinguish recent or chronic infections by discriminating antibodies avidity and titer. [9]

The current diagnostic algorithm relies on rapid antibody tests or ELISA as a preliminary screening in blood banks, followed by WB confirmation. A modified algorithm, which can
shorten the turnaround time and strengthen the sensitivity and specificity, was proposed in the 2010 HIV Diagnostics Conference with the devices described in the followings. [10] The 4th generation ELISA, that can simultaneously detect p24 antigens and both anti-HIV-1 and anti-HIV-2 antibodies, are now commonly being used in major resource-rich continents. [11-13] Several FDA-approved or CE-IVD kits are ARCHITECT HIV Ag/Ab Combo assay (Abbott Diagnostics, Germany), Enzygnost HIV integral II (Siemens Healthcare Diagnostics, Germany), GS HIV Combo Ag/Ab EIA (Bio-Rad Laboratories, USA) and VIDAS HIV DUO Ultra (bioMérieux, France). [14-17] In comparing to the traditional double-confirmed results by ELISA and WB, the 4th generation immunoassays can detect 84% of acute HIV infection and are >98% specific and sensitive. [11, 12] They can detect acute infections 7 days earlier than the 3rd generation ELISA (VITROS anti-HIV 1+2 assay, Ortho-Clinical Diagnostics, UK). [18] The NAAT-based qualitative assay, (APTIMA HIV-1 RNA Qualitative assay, Gen-Probe Inc., USA) can further reduce the window period to 26 days before western blot confirmation, due to the high level of viral replication before immune response establishment. [3, 18, 19] Rare false-positive results obtained by the NAAT assay had limited its first-line screening usage in Europe. [20, 21] HIV-1 detection can also be done by rapid tests, which are simple, faster and can be performed without intensive clinical or laboratory settings. The introduction of 2nd generation discriminatory rapid tests (Multispot HIV-1/HIV-2 rapid test, Bio-Rad...
Laboratories, USA) was proven to have comparable results against WB, although contradictory results were also reported. [22, 23] After evaluating both the pros and cons of these new technologies, the 4th generation ELISA assays (such as ARCHITECT Ag/Ab combo), were proposed to be used as the initial screening tool in the US and Europe[10, 21]. Any positive ELISA results will further be confirmed by Western Blot or HIV-1/HIV-2 discriminatory assay rapid test. The most sensitive and expensive NAAT tests (e.g. APTIMA) will only be used as a supplementary verification for any discordant detection.

**Viral load monitoring**

HIV-1 infections are considered as a chronic illness, and required non-stop antiretroviral therapy to suppress viral replication continuously. In order to maintain treatment efficacy, viral load, CD4+ counts and drug resistance mutations are monitored closely by different laboratory tests which will be discussed in the followings and summarized in Table 1.

Prior to viral load testing, sample preparation and RNA extraction are both crucial procedures for proper downstream processing. Blood samples are first collected in EDTA or plasma preparation tubes (PPT), followed by centrifugation to obtain plasma and/or peripheral blood mononuclear cells. Due to the instability of virus in specimen, storage under -70℃ are necessary. Yet the storage condition is impractical in remote-settings and for shipment after
plasma separation. [24] In some resource-limited countries, the use of dried blood spots (DBS) for sample collection has been proven to be able to keep the viral nucleic acid in good condition during transportation. The cost of using filter paper for DBS sampling is much more cost effective than using PPT or EDTA tubes for whole blood collection. [25] Using the Abbott HIV-1 Real-time assay (Abbott Molecular, USA), the RNA quantitative levels had no significant difference between freshly separated plasma or with DBS. In a small study cohort, DBS was 95% sensitive with respect to the real-time assays and the high concordance showed promising future on sample preparation. [25]

With a good sample collected, the next step would be viral nucleic acid extraction. Viral RNA extraction requires specialized equipments and sterilized reagents to prevent contaminations and RNA degradation. The procedure involves protein denaturation, RNA capture on solid silica surfaces, inhibitors removal and RNA elution from the silica. RNA becomes less stable after extraction, and requires ultra low temperature storage. Recently, a new device, RNAStable (Biomatrica, USA), was claimed to be able to stabilize RNA in a dry matrix form for at least 3 months under room temperature. [26] Apart from it, the trend of RNA extraction has switched from manual handling to automation in most developed countries. There are 3 commonly used CE-IVD marked automated nucleic acid extraction platforms, the Roche COBAS AmpliPrep system (Roche Molecular Diagnostics, Germany), the Abbott m2000
system (Abbott Molecular, USA) and the NucliSens easyMAG (bioMérieux, France) in the market, which can handle a wide range of biological samples with limited hands-on time. [27, 28] These fully automated RNA extraction systems provide standardized extraction protocols, which is important for extreme low-level viremia measurement. [29]

The level of plasma HIV-1 RNA can directly reflect the efficacy of HAART, the possibility of mother-to-child transmission, the odds of drug resistance mutations and the probability of AIDS progression. [30-32] In clinical definition, a successful ART treatment can inhibit viral replication and suppress the viral RNA level to ≤50 copies/ml after 24-week treatment. [33]

HIV-1 exists in different genotypes, unique and circulating recombinant forms in isolated continents. [6] A perfect viral load assay is therefore competent in identifying all the diverse genotypes and maintaining high sensitivity for substantial patient care. External Quality Assurance Programs (QCMD, CAP, NATA) are always in place for clinical diagnosis. A 10-year evaluation study (2000-2010) on an external quality assurance program in the United Kingdom revealed that end-point assays were gradually replaced by real-time assays. [29] In 2010, over 85% of the participating laboratories employed real-time assays for HIV-1 RNA quantification, which demonstrated the lowest coefficient of variation, most rapid turnaround time and highest throughout among the other methods.

Currently, there are several CE-IVD marked commercial assays used worldwide, together with
some in-house and research assays. These assays are based on nucleic acid sequence-based amplification (NASBA), branched-chain DNA assay (bDNA) and reverse transcription qualitative PCR assay (RT-qPCR). [34, 35] The NucliSENS EasyQ System HIV-1 QT test (bioMérieux, France) is the only assay using the NASBA technology. NASBA provides rapid real-time quantification by amplifying RNA with the use of isothermic heat-stable enzymes. [36] The updated version has allowed better sensitivity towards a range of non-B subtypes. [37] However, the EasyQ system was showed to have lower specificity and limits of detection (176 – 3,470,000 copies/mL) than other real-time PCR assays. [35, 38, 39] On the other hand, the VERSANT HIV-1 RNA 3.0 Assay (Siemens Healthcare Diagnostics, USA) uses the bDNA technology which relies on signal amplification of specific primer and probes binding to the HIV-1 pol region. Even though the bDNA assay was demonstrated to give higher diagnostic sensitivity; it performed poor in low viral load measurements and sometimes under estimated the viral RNA level in the specimens. [39] Its dynamic range is comparatively narrow, which is between 75 to 500,000 copies/mL only.

For Roche COBAS Amplicor HIV-1 Monitor Test (Roche Molecular Diagnostics, Germany), viral RNA was reverse transcribed into complementary double-stranded DNA, followed by standard PCR. The end-point assay is now gradually replaced by the more sensitive and faster real-time PCR assays. [29] In turns, the Abbott Real-Time HIV-1 system (Abbott Molecular,
USA) and the COBAS Taqman HIV-1 Test (Roche Molecular Diagnostics, Germany) are the currently leading technologies for HIV-1 viral load monitoring, with a wider dynamic diagnostic range of 40 - 10,000,000 copies/mL. [35] Both assays allow automated RNA extraction and adopting fluorescence-tagged probes targeting HIV-1 pol-int or gag gene respectively. These real-time assays apparently provide the best sensitivity and specificity on both B and non-B HIV-1 subtypes. [37]

Unfortunately, real-time quantitative assays are not readily available for resource-limited settings. The Cavidi ExaVir Load assay (Cavidi AB, Sweden) and the Ultra-Sensitive p24 Antigen Assay (Perkin Elmer Life Sciences, USA) do not require sophisticated laboratory set-up and provide moderate detection limits for viral load monitoring. The former assay estimates the reverse transcriptase activity manually while the later assay simply uses the ELISA approach. [40]

New possibilities are now shown to have lower quantitative limits beyond 50 copies/mL in real-time assays. The ultrasensitive VERSANT HIV RNA 1.0 assay (kPCR) (Siemens Healthcare Diagnostic, USA) was used to measure the virological response in a group of ART-experienced patients. The detection limit could reach 3 copies/mL. [41] However, the reproducibility of low vireamia is relatively variable by this assay, as well as the above-mentioned real-time assays by Abbott and Roche. [42] For instance, around 50% of the
blips could not be detected in one of the triplicate tests. Although these commercial tests can push the limit of detection to \( \leq 20 \) copies/mL, the reliability and stability remains a concern. It raised a question whether a single testing is appropriate in the future as biases between different commercial assays at low-level vireamia may affect treatment guidelines. Two or more consecutive viral load measurements should be considered to be more conclusive on treatment monitoring.

There have been controversial debates regarding the impact virologic blips; the persistent of HIV-1 RNA low vireamia at different categorized viral load copies will increase the chance of virological failure.\[41, 43-46\] The existing viruses can escape ART treatment, implying part of the viral population evolved under drug pressure and become drug resistant mutants. Various reasons, including ongoing viral replication, methodological variation or emergence of drug resistant viral particles, may explain the uncertain occurrence of blips. \[47\] Virological failure was observed in a significant high proportion of ART-experienced patients with viral load over 3 copies/mL, suggesting an update revision is required for the future treatment guidelines. \[41\] The relationship between blips and virological rebound or CD4+ decrease is still under investigation.

\[\text{CD4+ T lymphocyte enumeration}\]
In the last century, CD4+ cell count was used to guide the clinicians on the timing of the initiation of ART. To balance the benefits of early treatment and the economical burden, CD4+ cell counts of 500 cells/μL was updated as the standard level for treatment initiation instead of the previous 350 cells/μL. [48] Large collaborative studies had suggested the initiation of ART should be as soon as HIV-1 diagnosis regardless of CD4+ cell counts, which can effectively suppress HIV-1 transmission and AIDS progression. [33, 49] The CD4+ count level is also useful for treatment efficacy monitoring. Flow cytometry counting with fluorescent-labeled monoclonal antibodies is the most widely accepted choice in developed countries for enumeration. The only challenges come from the huge machines and high instrumental cost which makes it not applicable in resource-limited countries. Manufacturers developed various point-of-care CD4 testing devices utilizing limited infrastructure, are currently in-use in remote areas. For instance, the PIMA CD4 Analyzer (Alere, Germany), the Auto 40 System (Apogee Flow Systems, UK) and the PointCare NOW system were shown to have results as good as the traditional flow cytometer. [50-53] The Auto 40 system is as well validated with reference method and assessed with external quality control. [54] Hence, CD4+ counting become possible in rural countries for treatment monitoring.

Drug resistance monitoring (PIs, NRTIs and NNRTIs)
HIV-1 infected patients usually have their viral load and CD4+ counts monitored on a 3-month to 6-month basis in developed countries. Virological rebound or treatment failure is defined whenever the viral load is above 200 copies/mL or within the range of 50 to 200 copies/mL in two to three consecutive samples after 6 months of antiretroviral therapy. [33, 55] The failing condition may be due to poor drug adherence, adverse drug effects as well as the emergence of drug resistance mutants. [30] During each round of HIV-1 replication, the error-prone reverse transcriptase increases population dynamics by introducing random mutations into viral population. Certain proportion of the viruses may become fitter and survive under drug selective pressure. These viruses, carrying drug resistance mutations, will gradually accumulate and dominate the major population. Therefore, it is necessary to determine drug resistance mutations or in turns the drug susceptibility at the moment of virological rebound before switching treatment regimen.

Phenotypic and genotypic methods are both available commercially for drug resistance monitoring. Apart from clinical uses, both methods are vital for research and drug developments. For example, they can be used to deduce the viral resistance and drug inhibitory mechanisms. Phenotyping estimates the ability of *in vitro* viral entry or replication under drug pressure, with respect to a known susceptible reference strain. The *in vitro* assays require bio-safety class 3 level laboratory setting to handle infectious tissue cultures, cloning,
transfection and infection. Although phenotypic assays can provide more insights of the virus, the long turnaround time and expensive running cost restricted the usage to selected clinical cases only. [56] Genotyping, on the other hand, relies on gene amplification and direct sequencing, which can provide results within one week. The analysis of nucleic acid sequences can identify mutations that are established to have known phenotypic drug resistance. [57] However, genotyping cannot predict drug susceptibility directly and is rather difficult to interpret if the viral population is complex or super-infected. The basic principle of phenotyping is to monitor the viral replication and fitness under sequential antiretroviral drug concentrations. [56] This is achieved by direct isolation of viruses from human plasma or peripheral blood mononuclear cells, or by generation of a recombinant virus which carries viral sequences derived from clinical samples and a standard backbone genome. There are two major commercially available phenotyping tools for examining PIs and NRTIs/NNRTIs resistance. The PhenoSense HIV assay (Monogram Biosciences, USA) generates resistance test vectors by inserting the amplified protease (PR) and reverse transcriptase (RT) sequences into a modified HIV-1 NL4-3 molecular clone lacking PR and RT regions. The products will then be used to co-transfect human embryonic kidney 293 cell line with a luciferase expression vector to engineer a pseudotyped virus. The 293 cell line is later infected by the pseudotyped viruses under different concentrations of the antiretroviral drugs, and produce luciferase
proteins if replication succeeds. Luciferase activity can be measured in a quantification scale, so as to estimate the drug susceptibility. [58] The AntiVirogram (Virco BVBA, Belgium) is slightly different from PhenoSense. The recombinant virus generation procedures are similar. The downstream work relies on culturing the recombinant virus with human T cell line MT4 under all available antiretroviral drugs. No molecular cloning step is involved in this phenotyping assay and a panel of recombinant strains will be created to reflect the diversified viral population circulating in the patients. The assay compares the replicating capacity between the wild-type virus and the constructed virus to provide inhibitory concentration (IC$_{50}$) of the antiretroviral drugs. [59] Both assays can readily access the drug susceptibility of patient with viral load over 500 copies/mL. Although there are no significant differences between the two assays for PIs and NNRTIs resistances, it seems that the PhenoSense performs better than the Antivirogram in certain commonly used antiretroviral drugs such as Abacavir, Stavudine and Didanosine. [60] In comparing to phenotypic tests, genotypic tests provide a faster turnaround time and simpler workflow. Current genotypic tests involve direct sequencing of the viral PR and RT region. The protocols adopted by industries and research laboratories are similar; reverse transcription and amplification of the RNA extract, followed by population Sanger sequencing. The Trugene HIV-1 Genotyping Kit (Siemens Healthcare Diagnostics, USA) and the ViroSeq HIV-1
Genotyping System (Celera Diagnostics, USA) are both CE-IVD-marked in Europe and approved by the FDA in US. [61, 62] Many other commercial genotyping assays, such as GenoSure MG (Monogram, USA), and less-pricey in-house genotyping assays are also well evaluated worldwide. [63-66] The major limitation of both widely validated kits is that they were designed basing on the HIV-1 subtype B viral genome, whereas their performance on HIV-2 or other HIV-1 genotypes remains uncertain. A recent study showed that the sequencing primers of the ViroSeq system failed to sequence a panel of diverse subtypes. [67] In particular, 1 out of the 7 sequencing primers failed to sequence over 50% of the included non-B subtype samples. Since non-B subtype HIV-1 are the predominant circulating strains in Asia, Africa and some parts of the European continents [6], the high failure rate of the ViroSeq system on non-B viruses would be a major challenge in the future. It is believed that a modified version of primers will be released in order to provide better coverage to a wide range of genotypes identified recently. Independent laboratories have established various in-house genotyping targeting non-B subtypes, including subtypes A, C, D, CRF01_AE, CRF02_AG. The in-house assays have low sequencing failure rate and are able to achieve over 95% sensitivities and specificities against validated kits. [64, 68, 69] In combining the advantages of both genotyping and phenotyping, A third hybrid approach, the VircoTYPE HIV-1 (Virco BVBA, Beerse, Belgium), is comprised of genotyping technique but with phenotypic analysis.
It is a modified version of VirtualPhenotype-LM and uses a linear regression modeling with over 80,000 pairs of correlated genotypic and phenotypic samples for accurate drug susceptibility prediction. [59] This approach provides a third option for drug resistance monitoring by obtaining phenotypic information from genotyping only.

The interpretation of the genetic sequences relies heavily on the most updated knowledge of correlation between mutations and \textit{in vitro} drug susceptibility. There are several HIV-1 drug resistance algorithms available in the Internet, including the Stanford HIV db Program (http://hivdb.stanford.edu/) (Stanford University, USA) and the ANRS database (http://www.hivfrenchresistance.org) (Agence Nationale de Recherches sur le Sida, Paris, France). [70, 71] The former database allows the input of a single PR/RT mutation or the PR/RT sequence for drug resistance interpretation on 19 commonly prescribed PR and RT inhibitors while the later provides tables of rules for each class of drug resistance. Moreover, genotypic sequences rely heavily on manual proof-reading and interpretation to the occurrence of mixed viral population or poor sequence quality. This requires several hours of training for a new technical staff and it is difficult to standardize their interpretation level among laboratories.

A new automated sequence analysis tool, RECall (http://pssm.cfenet.ubc.ca), does not require manual editing and can identify mixed genetic population has been developed recently. [72] This analysis tool shared over 99% of sequence agreement in comparing to manual editing and
Drug resistance monitoring (Integrase and fusion inhibitors)

Integrase inhibitors (INI) and fusion inhibitors are the 2 recently FDA-approved antiretroviral drug classes. INI has a relatively low genetic barrier, and more expensive than PIs and NRTIs/NNRTIs. It is only used for patients who had developed multi-classes drug resistant or low tolerance of adverse effects. [33] Drug resistance monitoring is available for INI commercially, yet none of them were approved by the US FDA and CE-IVD marked. The basic principles of genotyping and phenotyping for INI and fusion inhibitors are similar to those of the PIs and RTIs classes. The PhenoSense and GeneSeq Integrase assays (Monogram, USA) are the more commonly used commercially available phenotyping and genotyping assay respectively. [73] Limited evaluation was carried on the ViroSeq and Trugene systems on their capability of integrase drug resistance interpretation. [74, 75] The testing on fusion inhibitor is further limited, which is mainly due to the inconvenient injections of fusion inhibitor. Moreover, natural occurring drug resistance is found in certain HIV-1 subtypes, restricting the susceptibility of this class. [76-78]

Tropism identification and drug resistance monitoring (CCR5 antagonist)
HIV-1 tropism is defined by the ability of virus infection with the two major chemokine co-receptors, CCR5 and CXCR4. [79] R5-tropic (R5) virus is previously thought to be the prevalent strain during transmission, while X4-tropic (X4) virus emerges due to AIDS progression at a later disease stage. [80, 81] Recent controversial studies observed a higher percentage of X4 virus in treatment-naïve patients of some subtypes and identified transmission cluster consisted of X4 virus only. [81-83] The transition phase in the viral population implies the possibility of a mixture of R5 and X4 viruses. The importance of tropism identification is growing, due to the first introduction of CCR5 antagonist into salvage therapy in 2007. Treatment guidelines in Europe and USA strongly recommend tropism tests must be done prior to initiation of CCR5 antagonist, as it only suppresses R5 viral entry. [33, 55, 84] The successfulness of CCR5 antagonist represents a new antiretrovirals era for scientists. The mechanism seems to be simpler than the traditional drug classes, although the side effects of blocking such co-receptor remain a concern in normal human metabolism. The phenotypic and genotypic tropism tests are therefore aggressively being developed in this decade. The MT-2 assay is the most traditional phenotypic tropism assay. Viruses isolated from patients are used to co-culture with human T cell line MT-2, which express CXCR4 coreceptors only. Syneytia will be formed if the viral isolates are able to infect MT-2 cells, implying the presence
of X4- or mixed/dual-tropic (D/M) viruses. [85] The use of MT-2 assay is limited, as it requires specialized laboratory set up and fresh samples for virus isolation. Apart from it, it is impossible to distinguish between virus isolation failure and a pure R5 virus population, due to the lack of a CCR5 coreceptor-expression cell line. To overcome the limitations in MT-2 assay, other single-cycle recombinant virus assays are as well applicable in tropism phenotypic tests.

[86] The Enhanced Sensitivity Trofile Assay (ESTA) (Monogram Biosciences, USA) is the current ‘gold standard’ assay that has been clinically validated the most. [87, 88] The amplified env gene is inserted into an expression vector, followed by co-transfecting 293 cell lines with a luciferase-expression vector. Quantification can be done by measuring the luciferase signal after a single round of infection of human primary glioblastoma U87 cell lines, with or without appropriate antagonists. The assay requires at least 1000 copies/ml of viral load to perform and 3 ml of fresh sample or frozen plasma that are stored in less than 3 months. As X4 virus usually exists as a minority, the detection limit of ESTA has now being improved greatly from the previous 10% to 0.3% of the total population and is 100% sensitive. [89] The Toulouse Tropism Test (INSERM, France) uses similar approach but with different backbone vector. Both phenotypic tests are highly concordant except the fact that the ESTA assay is more sensitive. [90]

Tropism determination can also be done by genotyping the third variable (V3) loop of HIV-1
The 35-amino acids region is believed to bind and interact with the co-receptor. The genotypic interpretation is originally based on the net charge and basic amino acids at position 11 and 25 of the sequences. Two more advance bioinformatic algorithms, such as Geno2Pheno [co-receptor] (G2P) 1.2 (http://coreceptor.bioinf.mpi-inf.mpg.de/index.php) and Web PSSM (http://indra.mullins.microbiol.washington.edu/webpssm), are publicly available and provide instant tropism predictions by V3 nucleotides or amino acids sequences respectively. G2P relies on the support vector machine technology trained with a large database of nucleotide sequences and corresponding phenotypes. The interpretation is given in the form of false positive rate, defining the likelihood of mistakenly classifying an R5 virus as X4 instead. Different cut-offs and clinical parameters can be chosen in G2P, depending on the patients’ treatment history and the amplification results. Web PSSM is slightly different, as it takes into account of every amino acid at every position, but not insertions and deletions, to determine the probability of an X4 virus. The interpretation is more complex when there is a mixed base pair positions and generate more than one answer, which make it less convenient for clinical practice and evaluation. Many clinical studies had reported a good correlation between Trofile and G2P genotyping data in subtypes B and C. Triplicate V3 sequencing is currently recommended, which may have a better chance of detecting the
Several limitations are observed in V3 genotyping. Some heavily-treated patients usually have a mixed viral population in their samples, which creates complication during direct sequencing. Population sequencing detects up to 20% of the minority, which means 20% of the hidden X4 virus can grow and dominate under a short period of CCR5 antagonist suppression. New technologies were developed recently to overcome these disadvantages. The denaturing heteroduplex tracking assay (HTA) can detect as low as 0.5% minority strains, which is more sensitive than Sanger sequencing. \([99, 100]\) R5 and X4 viruses may only differ in a single amino acid substitution. Therefore the HTA adopts various techniques to enhance the sensitivity and specificity. The V3 region is first amplified with locked nucleic acids incorporated primers, and annealed by a single-stranded fluorescent probe. The probe consists of V3 R5 consensus so that X4 samples can form heteroduplexes with the probe. A denaturing-gel-electrophoresis can distinguish the variants as DNA homoduplexes migrate faster than DNA heteroduplexes, whose conformation can be, strengthen by formamide. Viral tropic is therefore determined by the migration distance on the gel, and the study successfully detected viral quasispecies in over 50 clones. This technique opens a new door for molecular diagnosis in quantitative analysis and possible automation by the capillary electrophoresis system, another upcoming trend.
Ultra-deep pyrosequencing

Direct sequencing is only capable of detecting roughly 20% of the minor viral population, triggering more advance research to lower this detection limit. Ultra-deep pyrosequencing (UDS) technique, provided by the Genome Sequencer FLX (GS-FLX) and Junior (GS-Junior) systems (Roche-454 Life Sciences, Germany), has been developed to enhance the throughput and sensitivity for sequencing. The systems first generate a library by amplifying the target genes with specific fusion primer. Each library fragment will be attached to one bead, followed by emulsifying in a water-in-oil mixture inside microreactors. Emulsion PCR amplification creates millions of fragment copies which are then loaded onto the PicoTiterPlate device for pyrosequencing. [5, 101] The latest version can achieve up to 700 megabases throughputs within 23 hours with read length of 1,000 basepairs.

UDS has been extensively evaluated in HIV-1 diagnostic fields. Both pros and cons were reported from many clinical studies. First of all, the cost of running UDS is largely higher than population sequencing and is not as easy accessible as direct sequencing. More importantly, the error rate of UDS is very high comparing to direct sequencing. In turn, the high throughput is the major overwhelming advantage of UDS. Several PIs and RTIs resistance monitoring was carried by UDS lately. [102-104] It seems that UDS is more applicable to
treatment-experienced patients, yet more studies are required to support the use of UDS in clinical settings. Besides, the clinical response between the quasispecies and routine Sanger sequences was similar in a recent study. Any extra viral variants observed in quasispecies might actually do not exist in the population nor had loss of replicating ability. [102] Although the study was of a small group of patients, it pointed out that the new technology may not have many implications on clinical evaluation. Moreover, the large amount of data produced in UDS may require a more delicate and detailed database for analysis. Another retrospective study was conducted to show that UDS could predict the virological response more accurate than the triplicate tropism sequencing approach. [105] It included patients enrolled in MOTIVATE and A4001029 studies who were Maraviroc-experienced. Triplicate population sequencing was not able to accurately predict all X4-tropic infections. Any R5 predictions was further deep sequenced by GS-Junior or GS-FLX system, which have a sensitivity of 0.5% detection limit. A significant number of patients who were classified in R5 infection were re-grouped into D/M by UDS. The correlation between Maraviroc-responders from MOTIVATE and A4001029 studies was improved. The study showed that the tropism determined by UDS and ESTA were similar, suggesting UDS can potentially replace the necessity of phenotypic assay, and mark it as gold standard instead. The potential of replacing traditional Sanger sequencing by UDS in patient care require further evaluation on the cost and practicality.
Host genetics polymorphisms

The close interaction and relationship between virus, antiretroviral drugs and the host cannot be underestimated. It is because a few single nucleotide polymorphisms (SNPs) as well as human leukocyte antigen (HLA) typing are linked to clinical failure or hypersensitivity symptoms in HIV-1 patients. Therefore, understanding the link between pharmacogenomics and metabolism is crucial. The most important example is demonstrated in CCR5 polymorphisms. CCR5-\(\triangle\)32 has a 32-base deletion in CCR5 genes that will result in truncated malfunctioned protein. CCR5-\(\triangle\)32 homozygotes are naturally resistant to R5 infections and heterozygotes are expected to have a slower disease progression than normal. [106] In determining the host status of CCR5 gene, it helps clinicians to have a better idea on the frequency of treatment monitoring. A meta-analysis included over 12,000 genotyped study objects to evaluate the importance of CCR5-\(\triangle\)32 heterozygosity. [107] There were no consistent research outcome currently available, as contradicted predictions on the protective behavior of CCR5-\(\triangle\)32 remains unclear. The CCR5 level expressed in CCR5-\(\triangle\)32 heterozygotes can possibly be as high as normal, and the expression level can be affected by other factors apart from genotype.

Concerning the relationship of host genetic and drug hypersensitivity, HLA-B*5701 and
Abacavir is a well defined example. Abacavir is widely prescribed as the first line treatment regimen. Clinicians observed patients on Abacavir developed serious side effects, such as rash, fever, and these effects disappeared after discontinuing Abacavir treatment. Later it was found that if patients carrying HLA genoptype B*5701 in their alleles, they will have hypersensitivity reaction towards Abacavir. The prevalence of HLA-B*5701 varies greatly around the world, ranging from 8-10% in Caucasians and Thai, to 1% in Africans, and to nearly 0% in Japanese, Taiwanese and Korean. The treatment guidelines indicated that the screening of HLA-B*5701 is compulsory before Abacavir prescription. There are many FDA-approved HLA typing kits, which mostly utilize the direct sequencing techniques or make use of the specific oligonucleotide probes hybridization after PCR. Other SNPs were shown to have association with severe kidney tubular dysfunction in Tenofovir-experienced patients. Tenofovir is a popular first-line NRTI for treating HIV-1 infection with tolerable side effects usually. The renal clearance of Tenofovir involves multidrug-resistance protein 2 and 4, which are encoded by the adenosine triphosphate-binding cassette genes ABCC2 and ABCC4. Using the TaqMan SNP Genotyping Assays (Applied Biosystems, CA, USA) two SNPs of the ABCC2 gene were identified. The CC genotype at position -24 and AA genotype at position 1249, were shown to have strong association with kidney tubular dysfunction in Japanese and European population.
The highly polymorphic hepatic cytochrome P450 isoenzyme 2B6 (CYP2B6) gene demonstrates the last example of host genetic polymorphisms. This gene participates in many antiretroviral drugs metabolism, Efavirenz and Nevirapine in particular. [122] A SNP at position 516 that changes from guanine to thymidine on the CYP2B6, is widely reported to affect Efavirenz and Nevirapine concentration in plasma. [123] Direct sequencing can detect the SNPs easily, supplemented by pharmacokinetic studies to monitor the concentration of antiretroviral drugs in plasma. A new finding on the high Efavirenz level in hair, measured by liquid chromatography coupled with tandem mass spectrometry, provides more insights on alternative detection methods. [124, 125] The abovementioned examples elucidated the vital host genetic determinants affecting antiretroviral prescription preferences, together with the evidence on dissimilar disease progression. The cost of patient care after specific genes made known to the adverse side effects. Nonetheless, larger study cohorts are required to reveal the inconsistency in various SNPs and host reactions on virus and antiretroviral drugs.

**Conclusions & Future Perspectives**

After nearly three decades of the discovery of AIDS and HIV viruses, clinicians and scientists have gone through many hurdles in unmasking the mystery of this virus. Current diagnostic assays can detect both HIV antigens and antibodies, providing more rapid and faster detection
Viral load and CD4 measurements are crucial for treatment monitoring. Lowering the detection limit to possibly 20 copies/mL in viral load assays, the clinicians are able to identify treatment failure patients at the earliest stage. HIV-1 genotyping is widely accepted as the pre-dominant test to identify drug resistance mutations and tropism, although rare cases require phenotyping tools for detailed analysis.

It is not surprising that the detection limits, sensitivity and specificity, costing and turnaround time of all molecular assays will be improved this century with the introduction of new ideas like ultra-deep sequencing and nano-particles assays. Amplicons sequencing allow researchers to identify individual viral mutants that previously undetected in population sequencing. However, this technique is highly demanded in budgeting and infrastructure settings, and generates a large amount of data which requires highly-trained technicians and complicated softwares to analyze. Various constraints at resource-limited or point-of-care settings will as well be eliminated with portable devices with lower cost.
Executive Summary

HIV-1 detection

- HIV-1 diagnostic assays include p24 antigen test, enzyme-linked immunosorbent assays (ELISA), western blot (WB) and nucleic-acid amplification test (NAAT).
- The 4th generation of ELISA and newly developed NAAT could successfully detect HIV antigens and antibodies around 20 days earlier than WB confirmation.

Viral load monitoring

- Plasma preparation tubes and EDTA tubes are used for sample collection in developed countries, while dried blood spots are used in rural areas.
- RNA extraction is largely facilitated by automated systems to reduce hands-on time and provide standardized protocols.
- Traditional reverse transcriptase polymerase chain reactions are mostly replaced by real-time assays such as nucleic acid sequence-based amplification and branched-chain DNA assay.
- Low vireamia measurement, which may affect treatment guidelines, varies in between assays and laboratories, and requires further evaluation.

CD4+ T lymphocyte enumeration

- Flow cytometry counting is implemented in developed countries while mobile flow
cytometers provide alternative measurements in rural countries.

**Drug resistance monitoring (PIs, NRTIs and NNRTIs)**

- When a patient experiences virological rebound or CD4+ decline, viral phenotyping and genotyping is required to estimate drug susceptibility and resistance.
- Phenotyping relies on the cloning of protease and reverse transcriptase sequences into a modified vector, which can be transfected into human embryonic kidney cell line. Luciferase signals are generated when the pseudotyped virus manage to infect the cell line again or co-culture with human T cell line and replicate under different concentrations.
- Genotyping bases on direct sequencing of protease and reverse transcriptase regions and analyzed by commercial phenotyping database or various open-assessed algorithms on internet.

**Drug resistance monitoring (Integrase and fusion inhibitors)**

- Phenotyping and genotyping assays are similar to those for PIs, NRTIs and NNRTIs.
- Due to the less common use of integrase and fusion inhibitors, limited clinical validation is available.

**Tropism identification and drug resistance monitoring (CCR5 antagonist)**

- HIV-1 virus utilizes CCR5 and/or CXCR4 co-receptor for viral entry. It is compulsory to identify viral tropism before the use of CCR5 antagonist.
Viral tropism can be determined by phenotyping and genotyping, with similar principles in pol gene.

Currently, no known CCR5 antagonist drug resistance mutations are identified.

**Ultra-deep pyrosequencing**

In comparing to Sanger sequencing, ultra-deep pyrosequencing can detect up to 0.5% of minor variants in viral population.

Clinical studies demonstrated better treatment guidelines by ultra-deep pyrosequencing.

However, the machines and running costs are extremely high that restrict the possibility of routine monitoring in viral load, drug resistance mutations and tropism identification.

**Host genetics polymorphisms**

A few single nucleotide polymorphisms and human leukocyte antigen (HLA) typing were shown to have clinical relevance on treatment failure and hypersensitivity reactions in HIV-1 patients.

Examples of CCR5-Δ32, HLA-B*5701, ABCC2 gene and CYP2B6 are discussed.
### Table 1. Summary of current diagnostic assays

<table>
<thead>
<tr>
<th>Current Technology</th>
<th>Target Sites</th>
<th>Most Common Assays</th>
<th>Manufacturers</th>
<th>Detection Limits</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HIV-1 Detection</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>ELISA (4th generation)</td>
<td>HIV-1 &amp; HIV-2 Ab &amp; p24 Ag</td>
<td>ARCHITECT HIV Ag/Ab Combo assay*</td>
<td>Abbott</td>
<td>p24: &lt; 50 pg/mL&lt;br&gt;Ab: 100% Sensitive 20 days before WB +ve</td>
<td>10-14</td>
</tr>
<tr>
<td></td>
<td>HIV-1 gp41, HIV-2 gp36 Ab &amp; HIV-1 p24 Ag</td>
<td>Enzygnost HIV Integral II</td>
<td>Siemens</td>
<td>p24: &gt;100 pg/mL&lt;br&gt;Ab: 100% Sensitive 14 days before WB +ve</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HIV-1 gp160, HIV-2 gp36 Ab &amp; p24 Ag</td>
<td>VIDAS HIV DUO Ultra</td>
<td>bioMérieux</td>
<td>p24: &gt;3 pg/mL&lt;br&gt;Ab: &gt;98% Sensitive 20 days before WB +ve</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HIV-1 gp160, HIV-2 env Ab &amp; HIV-1 p24 Ag</td>
<td>GS HIV Combo Ag/Ab EIA*</td>
<td>Bio-Rad</td>
<td>p24: &lt; 50 pg/mL&lt;br&gt;Ab: 100% Sensitive 19 days before WB +ve</td>
<td></td>
</tr>
<tr>
<td>NAAT</td>
<td>RNA</td>
<td>APTIMA HIV-1 RNA Qualitative*</td>
<td>Gen-Probe</td>
<td>RNA: &gt;14 cp/mL&lt;br&gt;95% Sensitive 26 days before WB +ve</td>
<td>16</td>
</tr>
<tr>
<td><strong>Viral Load Monitoring</strong></td>
<td>RNA gag &amp; LTR region</td>
<td>COBAS Tagman HIV-1*</td>
<td>Roche</td>
<td>48 – 10,000,000 cp/mL</td>
<td>31, 33</td>
</tr>
<tr>
<td></td>
<td>RNA pol</td>
<td>Abbott Real-time HIV-1*</td>
<td>Abbott</td>
<td>40 – 10,000,000 cp/mL</td>
<td></td>
</tr>
<tr>
<td>NASBA</td>
<td>RNA gag</td>
<td>NucliSENS EasyQ system HIV-1 QT*</td>
<td>bioMérieux</td>
<td>176 – 3,470,000 cp/mL</td>
<td>31-34</td>
</tr>
<tr>
<td>bDNA</td>
<td>RNA gag</td>
<td>Versant HIV-1 RNA 3.0 *</td>
<td>Siemens</td>
<td>75 – 500,000 cp/mL</td>
<td>35</td>
</tr>
<tr>
<td>RT-kPCR</td>
<td>RNA pol/int</td>
<td>Versant HIV RNA 1.0</td>
<td>Siemens</td>
<td>37 – 11,000,000 cp/mL</td>
<td>37</td>
</tr>
<tr>
<td><strong>Drug Resistance Monitoring</strong></td>
<td>RNA pol (PR &amp; RT)</td>
<td>Phenosense</td>
<td>Monogram</td>
<td>≥ 500 cp/mL</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>RNA pol (INT)</td>
<td>Phenosense Integrase</td>
<td>Monogram</td>
<td>Limited information available</td>
<td>54</td>
</tr>
<tr>
<td>Phenotyping (cloning, transfection &amp; infection)</td>
<td>RNA pol (PR &amp; RT)</td>
<td>Trugene*</td>
<td>Siemens</td>
<td>≥1,000 cp/mL</td>
<td>56</td>
</tr>
<tr>
<td>Genotyping (direct sequencing)</td>
<td>RNA pol (INT)</td>
<td>Viroseq*</td>
<td>Celera</td>
<td>2,000 – 750,000 cp/mL</td>
<td>57</td>
</tr>
<tr>
<td>Phenotyping (cloning, transfection &amp; infection)</td>
<td>RNA pol (gp160)</td>
<td>Enhanced Sensitivity Trofile Assay</td>
<td>Monogram</td>
<td>100% Sensitive at 0.3% CXC4R4, ≥1,000 cp/mL</td>
<td>82</td>
</tr>
<tr>
<td>Tropism Determination</td>
<td>RNA env (gp120 &amp; gp41)</td>
<td>Toulouse</td>
<td>INSERM</td>
<td>100% Sensitive at 0.5% CXC4R4, ≥1,000 cp/mL</td>
<td>83</td>
</tr>
<tr>
<td>Genotyping (direct sequencing)</td>
<td>RNA env (V3 loop)</td>
<td>In-house only</td>
<td>----</td>
<td>----</td>
<td>77, 84, 85</td>
</tr>
</tbody>
</table>

**Abbreviations:** * - FDA approved assays; ELISA – Enzyme-Linked Immunosorbent Assay; NAAT – Nucleic Acid Amplification Test; WB – Western Blot; RT-qPCR – Reverse Transcriptase – qualitative Polymerase Chain Reaction; NASBA – Nucleic Acid Sequence-Based Amplification; Ab – Antibodies; Ag – Antigens; bDNA – Branched-chain DNA assay; RT-kPCR – Reverse Transcriptase – kinetic Polymerase Chain Reaction; cp/mL – copies/mL; +ve – positive; LTR – Long-Terminal Repeats; PR – protease; RT – Reverse Transcriptase; INT - Integrase
References


15. Bentsen C, McLaughlin L, Mitchell E *et al.*: Performance evaluation of the Bio-Rad Laboratories GS HIV Combo Ag/Ab EIA, a 4th generation HIV assay for the simultaneous detection of HIV p24 antigen and antibodies to HIV-1 (groups M and O) and HIV-2 in human


28. Gueudin M, Plantier JC, Lemee V et al.: Evaluation of the Roche Cobas TaqMan and


* Good overview on viral load measurement among Europe laboratories.


34. Puren A, Gerlach JL, Weigl BH, Kelso DM, Domingo GJ: Laboratory operations, specimen processing, and handling for viral load testing and surveillance. *The Journal of*
infectious diseases 201 Suppl 1, S27-36 (2010).


42. Ruelle J, Debaisieux L, Vancutsem E et al.: HIV-1 low-level viremia assessed with 3 commercial real-time PCR assays show high variability. *BMC Infect Dis* 12, 100 (2012).


*More evidences were provided to emphasize the importance of vireamia blips on treatment
failure patients.


53. Larson BA, Schnippel K, Ndibongo B et al.: Rapid Point-of-Care CD4 Testing at Mobile


59. Pattery T, Verlinden Y, De Wolf H *et al.*: Development and performance of conventional HIV-1 phenotyping (Antivirogram(R)) and genotype-based calculated phenotyping assay (virco(R) TYPE HIV-1) on protease and reverse transcriptase genes to evaluate drug resistance.


immunodeficiency virus-1 genotyping systems: ViroSeq 2.0 and an in-house method. *J Virol* 760


67. Aghokeng AF, Mpoudi-Ngole E, Chia JE, Edoul EM, Delaporte E, Peeters M: High
failure rate of the ViroSeq HIV-1 genotyping system for drug resistance testing in Cameroon, a

HIV-1 protease and reverse transcriptase drug resistance genotyping of all major group-M

69. Nie T, Detorio M, Schinazi RF: Universal profiling of HIV-1 pol for genotypic study and

70. Eberle J, Gurtler L: The evolution of drug resistance interpretation algorithms: ANRS,
REGA and extension of resistance analysis to HIV-1 group O and HIV-2. *Intervirology* 55(2),
128-133 (2012).

71. Tang MW, Liu TF, Shafer RW: The HIVdb system for HIV-1 genotypic resistance


73. Fransen S, Gupta, S., Paxinos, E. E., Huang, W., Parkin, N., Miller, M., Hazuda, D.,
Petropoulos, C. J.: Integrase (IN) Inhibitor Susceptibility Can Be Measured Using Recombinant Viruses That Express Patient Virus IN Alone, or in Combination with Protease (PR) and Reverse Transcriptase (RT). *12th Conference on Retroviruses and Opportunistic Infections, February 2005, Boston,*


85. Louder MK, Mascola JR: Determination of Syncytium-Inducing Phenotype of Primary


91. Briggs DR, Tuttle DL, Sleasman JW, Goodenow MM: Envelope V3 amino acid sequence predicts HIV-1 phenotype (co-receptor usage and tropism for macrophages). *Aids* 14(18),
2937-2939 (2000).


Lataillade M, Chiarella J, Yang R et al.: Virologic failures on initial boosted-PI regimen infrequently possess low-level variants with major PI resistance mutations by ultra-deep


201. 2011 Global epidemic data and statistics, Geneva, World Health Organization

http://www.who.int/hiv/data/en/