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Improving Clinical Laboratory Efficiency: Introduction of Systems for the Diagnosis and Monitoring of HIV Infection

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Abstract: Since the first tests for identifying individuals with suspected human immunodeficiency virus (HIV) infection were introduced in the mid-1980s, diagnostic virology testing has greatly evolved. The technological advances, automating in the laboratories and the advances in molecular biology techniques have helped introduce invaluable laboratory methods for managing HIV patients. Tests for diagnosis, specially for screening HIV antibodies, are now fully automated; in the same way, tests for monitoring HIV viral load (HIV RNA copies/ml of plasma), which is used for monitoring infection and response to antiretroviral treatment, are also fully automated; however, resistance testing, tropism determination and minor variant detection, which are used to make decisions for changing antiretroviral treatment regimens in patients failing therapy, still remain highly laborious and time consuming. This chapter will review the main aspects relating to the automating of the methods available for laboratory diagnosis as well as for monitoring of the HIV infection and determination of resistance to antiretrovirals and viral tropism.

Keywords: Diagnosis, hiv infection, monitoring, resistance testing, viral load, tropism.

INTRODUCTION

Since the first tests for identifying individuals with suspected human immunodeficiency virus (HIV) infection were introduced in the mid-1980s, diagnostic virology testing has greatly evolved thanks to increased understanding of immunopathogenic mechanisms, the host-virus relationship, viral replication mechanisms and the immune response that occurs in infected people over the course of the infection.

The technological advances, automating in the laboratories and the advances in molecular biology techniques have helped introduce invaluable laboratory methods for managing HIV patients. During the course of HIV infection, various viral markers can be used to identify the infection and monitor the treatment. Each has different kinetics and appears at a different time and so the choice of marker to be detected will depend on the objective of the diagnosis [1]. The first marker to appear after infection is HIV RNA, which can be detected by amplification techniques around 2 weeks after infection, generally at 10-12 days. At more or less the same time as the HIV RNA, HIV DNA integrated into the cell genome (proviral DNA) can be detected. The p24 antigen appears in serum at 11-13 days and can be detected using maximum-sensitivity techniques for about one and a half months [2]. Antibodies can be detected in serum at 3-4 weeks after infection (average 22 days), reaching peak concentration at 10-12 weeks [3]. When the antibodies appear, the viraemia levels diminish and the p24 antigen disappears as a result of the formation of immunocomplexes [1]. The interval between infection and the appearance of antibodies or seroconversion is known as the window period and is characterised by the presence of HIV RNA, proviral DNA, and p24 antigen and the absence of specific antibodies.

Laboratory diagnosis of HIV infection is mainly done through demonstration of the presence of anti-HIV antibodies, for which screening and confirmation techniques have to be used [4]. Determination of proviral DNA is used at times, primarily for diagnosis of vertically-transmitted infection. Once HIV infection is confirmed, HIV viral load (HIV RNA expressed in copies/ml of plasma) and resistance testing are used to evaluate the efficacy of antiretroviral therapy, and to design new treatment regimens in patients with treatment failure. A new tool to help design the new treatment regimen is the determination of viral tropism.

This chapter will review the main aspects relating to the automating of the methods available for laboratory diagnosis as well as for monitoring of the HIV infection and determination of resistance to antiretrovirals and viral tropism.

DIAGNOSIS OF HIV INFECTION

HIV Infection Detection Techniques

Infections are diagnosed by detecting the presence of specific antibodies, since they are found in the serum in virtually 100% of infected people. With the aim of minimising the risk of false-negative results, all the techniques are highly sensitive, with close to 99% accuracy. It is difficult to achieve 100% sensitivity as seroconversion does not take place until 3-4 weeks after infection and, moreover, infected people can be seronegative as a result of immunity defects. The increase in sensitivity entails a reduction in specificity, which with the current techniques is around 99%. Furthermore, a lower prevalence of HIV

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infection in the studied population reduces the positive predictive value, meaning that the likelihood of false-positive results occurring with low HIV infection rates is higher. All positive results must therefore be confirmed by a confirmatory test [1].

Screening Techniques

ELISA. The first techniques were introduced in diagnosis in 1985 but they have greatly evolved since then. Now, third-generation ELISA are used with "sandwich" format which detect IgG and IgM antibodies and antibodies to all the M subtypes, groups N and O and HIV-2. Fourth-generation techniques have recently been introduced which allow simultaneous detection of antibodies and p24 antigen, reducing the window period to 13-15 days [5]. With these techniques, the sensitivity is increased to 99.9%. This reduces the likelihood of a false-negative result and means that in principle, a negative result does not require either confirmation or serological monitoring except in people at high risk of acquiring the infection [6]. The specificity is between 99.5% and 99.9%. However, false positives can occur as a result of non-specific recognition of substances in the serum by the virus antigens from the antigen base.

ELFA (enzyme linked immunofluorescent assay). These tests are modified versions of the ELISA techniques that use solid phases with a larger contact surface area, thereby reducing incubation times. As label, they use an enzyme and fluorescent substrates which are transformed into a fluorescent product by the action of the enzyme. The rates of fluorescence emitted are measured with a fluorescence detector. These techniques enable the process to be automated, the processing of a large number of samples, less handling of the sample (which reduces the number of errors), a reduction in costs and, most importantly, a reduction in the duration of the process, with first result response times of less than 60 minutes [7]. Third- and fourth-generation techniques are both currently used.

Chemiluminescence. These techniques use chemiluminescent compounds to label the antigens or antibodies which, when put in contact with a substrate and an oxidant, form an unstable intermediate compound. When this returns to its ground state, it becomes excited and emits energy in the form of light which is measured by a photomultiplier tube. Various chemiluminescent compounds can be used, such as luminol, dioxetane, ruthenium and acridine. These techniques have been adapted to large analysers and have many advantages as: they are totally automated; primary tubes with barcode can be used; reagents can be kept refrigerated; long stability of the calibration curve; 200-400 tests/hour; response time of 30-60 minutes; they make it possible to design conditional test methods; and bidirectional computer connection is possible. Third- and fourth-generation methods are currently available.

Confirmatory Tests

The most commonly-used confirmatory techniques are Western Blot (WB) and recombinant immunoblot or line immunoassay (LIA). They have at least the same sensitivity as ELISA and superior specificity. Both techniques can incorporate HIV-2 envelope antigens, thereby making it possible to diagnose HIV-2.

The methodology for both methods has now been semi-automated, making them easier to perform. However, the results can be subjective as the reading is based on observation of the presence of coloured bands which correspond to different viral proteins [7]. Each laboratory must therefore establish a strict system for taking reactivity readings.

Detection of Antigenaemia

The detection of specific antibodies indicates exposure to the virus and infection and direct detection of the viral p24 antigen introduces a dynamic concept to serology; since it is a marker of virus replication, it provides information on the current status of the infection, it is detected in initial stages of the infection or in the development of AIDS, and it serves as back-up to the serological diagnosis in situations where the detection of antibodies is inconclusive [8]. The p24 antigen can be measured in serum and plasma with uptake ELISA techniques that increase sensitivity, which at present can be as high as 8 pg/ml.

The main characteristics of the available systems for screening and confirmation of HIV infection and the extent to which they are automated are shown below. Table **1** shows the methodology, generation, format and main characteristics of fully automated systems for HIV diagnosis. Table **2** lists the main features of ELISA semi-automated systems. Table **3** shows the confirmatory assays and their features.

VIROLOGICAL MARKERS FOR MONITORING HIV INFECTION

HIV Viral Load

Viral load, or plasma viraemia, is the amount of virus circulating in plasma and refers to the number of copies of HIV RNA present per millilitre, expressed as copies/ml or $\log_{10}$. The HIV viral load and CD4 lymphocyte count are currently the two most used prognostic markers of clinical progression of the HIV infection [9]; the first is an indicator of virus replication and the second reflects how the host's immune system is working. Both parameters are monitored every 3-6 months, although some authors [10] are now advocating annual monitoring, at least of the CD4 count. A high initial viral load is a marker of rapid progression and clinical progression is always preceded by an increase in the viral load. Changes in plasma HIV-1 RNA levels are the best marker for response to antiretroviral therapy, since they are sensitive, fast and reliable in terms of good management of the patient with HIV infection. The success of antiretroviral therapy is defined in the majority of guidelines as the suppression of plasma viraemia and clinically, it is considered as such when the patient has two successive viral loads below 50 copies/ml [11], although this cut-off point has been the subject of debate in the last year [12-14].

Viral load should not be used routinely to diagnose the infection, except in special cases such as confirmation of neonatal infection.

Given how important it is to measure this parameter, accurate, reproducible, cost-effective techniques are required [15-17].

Table 1. Automated Antibody Detection Techniques

Vendor	System	Marker	Method/Generation/Format	Characteristics
ABBOTT	ARCHITECT	Antibodies + Antigen	Chemiluminescence (CMIA)/ 4th generation/Sandwich	Modular system; 200-800 determinations/hour Primary tube and aliquot / barcode 25 refrigerated reagents per module / stable 30 days / 5 hours of autonomy 135 samples / module / continuous loading / immediate emergency processing (35 positions/module) Response time 29 minutes Sample volume 150 ul / automatic repetitions / conditional tests Pressure differential clot detection Intelligent wash system. Minimal carry-over (1 ppm) Reagents with barcode: Information on batch, expiry date, calibration curves Long calibration curve stability Automatic internal quality control system (Levey-Jennings) Remote diagnostics (AbbottLink™). Connection to Laboratory Information System (LIS)
ROCHE	COBAS	Antibodies + Antigen	Chemiluminescence (ECLIA)/ 4th generation/Sandwich	Modular system; 88-170 determinations/hour Primary tube and aliquot / barcode 18-25 refrigerated reagents per module 100-150 samples / module / continuous loading / immediate emergency processing Response time 18 minutes Sample volume <50 ul /automatic repetitions. Conditional tests Disposable tips and cups that prevent contamination Reagents with barcode: Information on batch, expiry date, calibration curves Calibration stable 28 days. Connection to Laboratory Information System (LIS)
IZASA	ACCESS 2 Unicel™ DxI 400-800	Antibodies + Antigen	Chemiluminescence/ 4th generation/Sandwich	Modular system; 200-400 determinations/hour Primary tube / automatic taking of aliquots and release of primary tube in 5 minutes / barcode 50 refrigerated reagents per module / stable 30 days / autonomy 1200 tests 120 samples / module / continuous loading / immediate emergency processing Response time 55; Sample volume <50 ul / automatic repetitions / conditional tests Fixed tip, no carry-over, wash system with carry-over <10 ppm Reagents with barcode: Information on batch, expiry date, calibration curves Calibration curve stability 26 days Connection to Laboratory Information System (LIS)
DIASORIN	LIAISON XL	Antibodies + Antigen Separate	Chemiluminescence (CLIA)/ 4th generation/Sandwich	171 determinations/hour Primary tube / barcode 250 refrigerated reagents per module / stable 8 weeks 120 samples / continuous loading / immediate emergency processing Response time 40 minutes Sample volume 350 ul / automatic repetitions / conditional tests Reagents with barcode: Information on batch, expiry date, calibration curves Calibration curve stability 15-30 days Connection to Laboratory Information System (LIS) Disposable tip / no carry-over / clot detection Independent antigen and antibody signals / sensitivity of p24 Ag: 1.26 IU/ml
SIEMENS	CENTAURO XP and CP	Antibodies	Chemiluminescence/ 3rd generation/Sandwich	Disposable tips. Capacity for 840 tips simultaneously Incubation time 58 min Sample volume 50 ul Calibration every 21 days, on-board stability 42 days
		Antibodies +Antigen	Chemiluminescence/4th generation/Sandwich	Disposable tips. Capacity for 840 tips simultaneously Response time 58 min Sample volume 100 ul Calibration every 21 days, on-board stability 42 days Sensitivity p24 Ag 1.15 IU/ml

There are currently a number of different platforms available for quantifying HIV viral load:

- Abbott RealTime HIV-1 m2000rt (Abbott Molecular Diagnostics): combines the extraction in the automated m2000sp system and qrtPCR amplification and detection with the automated m2000rt equipment.
- COBAS AmpliPrep TaqMan HIV-1 48 (Roche Molecular Diagnostics): uses the COBAS AmpliPrep for the extraction of the sample and the COBAS TaqMan 48 v2.0 for the qrtPCR amplification and detection.
- NucliSens EasyQ HIV-1 v1.2 (bioMérieux): uses the EasyMAG for the extraction and the EasyQ for qrtPCR amplification and detection.

Table 2. Semi-Automated Antibody Detection Techniques

Vendor	System	Marker	Method	Characteristics
BIORAD	EVOLIS	Antibodies	ELISA/3rd generation/Sandwich	Response time: 2 h Can be fully automated on EVOLIS Connection to Laboratory Information System (LIS)
		Antibodies + Antigen	ELISA/4th generation/Sandwich	Response time: 2 h 30 min Analytical sensitivity for HIV Ag: 13.6 pg/ml Can be fully automated on EVOLIS Connection to Laboratory Information System (LIS)
		p24 antigen	ELISA/Sandwich	Response time: 3 h Can be fully automated on EVOLIS
IZASA	BEST 2000	Antibodies	3rd generation HIV ELISA	Response time: 2 h Can be fully automated on Best 2000 or any analyser Connection to Laboratory Information System (LIS)
		Antibodies + Antigen	4 generation ELISA	Response time: 2 h 30 min Can be fully automated on Best 2000 or any analyser Connection to SIL
DIA SORIN	ETI-MAX	Antibodies	3rd generation ELISA/Sandwich	Response time: 2 h Can be fully automated on Eti-Max. Connection to Laboratory Information System (LIS)
		Antibodies +Antigen	ELISA/4th generation/Sandwich	Response time: 2 h 30 min Analytical sensitivity for HIV Ag: 16 pg/ml Can be fully automated on Eti-Max. Connection to Laboratory Information System (LIS)
SIEMENS	BEP III, BEP 2000	Antibodies	ELISA/3rd generation/Sandwich	Response time: 3 h Can be fully automated on Bep III/2000. Connection to Laboratory Information System (LIS) Disposable tips
		Antibodies +Antigen	ELISA/4th generation/Sandwich	Response time: 3 h Can be fully automated on BepIII/2000, Connection to SIL Disposable tips Bidirectional connection Analytical sensitivity between 100 and 250 pg for p24 Ag

Table 3. Confirmatory Assays

Manufacturer	System	Marker	Method	Characteristics
IZASA	Autoblot	Confirmatory of HIV-1/2	Western-Blot	Antigen base: all the HIV-1 proteins and HIV-2 gp36 and an internal control Incubation time: 3 h Response time: 3 h 30 min
INNOGENETICS	Autoblot	Confirmatory of HIV-1/2	Immunoblot	Antigen base: HIV-1: gp120 (subtype 0), gp41, p31, p24 and p17/ HIV-2: gp105, gp36. Internal control Incubation time: 3 h Response time: 3 h 30 min
BIORAD	Autoblot	Confirmatory of HIV-1	Western-Blot	Antigen base: all the HIV-1 proteins and an internal control Incubation time: 3 h Response time: approx. 3 h 30 min
	Autoblot	Confirmatory of HIV-1 and HIV-2	Immunoblot	Antigen base: gp41 and gp36 Incubation time: 1 h 30 min Response time: approx. 2 h

- VERSANT HIV-1 RNA 1.0 (kPCR) (Siemens): this system combines a sample extraction module (SP module) with an amplification and detection module (AD module).
- Artus HIVirus-1 QIAsymphony SP/AS and Rotor-Gene Q (Qiagen): the extraction is carried out in the QIAsymphonySP/AS equipment and the amplification and detection in the Rotor-Gene Q.

All of these use real-time PCR to quantify HIV RNA in plasma, displacing previously-used techniques such as conventional PCR, the NASBA technology, ligase chain reaction or signal amplification. The advantages of real-time PCR are its high levels of analytical sensitivity, reproducibility and linearity, dynamic range and the fact that the current techniques can quantify different types and subtypes of HIV, not only HIV-1 subtype B. These instruments incorporate

automated RNA extraction methods which significantly reduce the time it takes to obtain results and the risk of contamination, and a larger number of samples can be processed. They all start with the reverse transcription of HIV-1 RNA to cDNA. The target is then amplified with real-time PCR, generating fluorescent molecules which are ligated to oligonucleotide probes which specifically bind to the amplified product. When the fluorescence exceeds a minimum signal, the number of PCR cycles (Ct) that have taken place is used for the quantification. One of the advantages of this technology is that it enables detection and quantification of the target without having to handle the reaction tubes. The main features of these assays are shown in Tables **4-7**.

There is intrinsic variability between the different assays and it is therefore recommended that the monitoring of a patient's viral load is always done with the same system; if a change of assay is unavoidable, it must be ensured that a new baseline quantification of the HIV-1 RNA is obtained with the technique to be used thereafter. For quantification in the different subtypes of HIV, nowadays, all these techniques have been adapted to detect a large number of non-B subtypes.

Resistance Testing

Drug resistance testing has become a key component of proper HIV clinical care and is currently recommended by most HIV treatment guidelines [11, 18, 19] as the standard of care, both in terms of choosing the most effective antiretroviral therapy for first-line regimens, by investigating the transmission of drug-resistant variants at the time of diagnoses (primary resistance) and in terms of the selection of active drugs in subsequent failures to first- or further-treatment-line failures (acquired resistance).

Although resistance assays can be categorized as either phenotyping or genotyping, the genotyping assays are the most widely-adopted in routine diagnostic laboratories. Phenotyping resistance assays measure the extent to which an antiretroviral drug inhibits virus replication *in vitro* and is usually performed by demonstrating a change in the inhibitory concentration (IC) that is required to inhibit *in vitro* growth by 50 percent (IC50) compared with virus replication in the absence of drug. Results are reported as a fold change in drug susceptibility of the patient sample in relation to a wild type reference strain. These assays are currently performed using Recombinant Virus Assay technology. They are typically available from commercial laboratories and are rarely performed in routine diagnostic laboratories. An overview of the phenotyping methods and their advantages and limitations can be found in Garcia *et al.* [4].

Genotyping resistance assays detect the presence of specific drug-resistance mutations. Mutations are then interpreted into a drug-resistance report by using resistance

Table 4. Target Genomic Region, Characteristics of the Internal Control, Amplification and Detection Strategies, Results Reports, Decontamination System, Dynamic Range, Specificity and Subtype Detection Features Provided by the Main Manufacturers of Commercial Viral Load Assays

	Abbott RealTime HIV-1 (m2000rt)	**COBAS® AmpliPrep/COBAS® TaqMan HIV-1, v2.0 (Roche)**	**NucliSens® EasyQ HIV-1 v1.2 (bioMérieux)**	**VERSANT® HIV-1 RNA 1.0 (kPCR) (Siemens)**	**Artus HI Virus-1 QS-RGQ (Qiagen)**
HIV target region	Highly conserved region within the *pol* gene (integrase)	Dual target: highly conserved region of the *gag* gene (p41) and LTR	Highly conserved region within the *gag* gene (p24)	Highly conserved region within the *pol* gene (integrase)	5´LTR region
Internal control	Yes. Non-competitive	Yes	Yes. Non-competitive	Yes	Yes
Amplification	Real-time PCR	Real-time PCR (TaqMan)	Real-time PCR (NASBA)	Real-time PCR (TaqMan)	Real-time PCR
Detection	Fluorescence	Fluorescence	Fluorescence - Molecular beacons	Fluorescence	Fluorescence
Quantification	Copies/ml, $\log_{10}$ copies/ml, IU/ml or $\log_{10}$ IU/ml; conversion factor to IU/ml is 1 IU=0.56 copies and 1 copy=1.74 IU	Copies/ml, $\log_{10}$ copies/ml; conversion factor to IU/ml is 1 IU=0.6 copies and 1 copy=1.7 IU	Copies/ml; conversion to IU 1:1	Copies/ml; IU/ml	UI/ml; conversion factor 1 IU=0.46 copies and 1 copy=2.17 IU
Linear dynamic range	40 copies/ml from 600 µl 11 million copies/ml	20 copies/ml from 850 µl 10 million copies/ml	24 copies/ml 1 million copies/ml	37 copies/ml 11 million copies/ml	112.5 copies/ml 45 million copies/ml
Specificity (%) (95%)	100 (99.28-100)	100 (99.3-100)	100	99.7 (99.3-100)	100
Subtypes/HIV-2	Group M -subtypes A-D, F-H, various CRFs, including CRF01_AE and CRF02_AG; group N, O and P. Does not detect HIV-2.	Group M -subtypes A-D, F-H, various CRFs, including CRF01_AE; group O Does not detect HIV-2.	Group M -subtypes A-D, F-H, J Does not detect HIV-2.	Group M -subtypes A-D, F-H, CRF01_AE and CRF02_AG; group O Does not detect HIV-2.	Group M -subtypes A-D, F-H Does not detect HIV-2.
Control of contamination with AmpErase	No	AmpErase (UNG)	No	AmpErase (UNG)	No

Table 5. Type of Samples, Sample Volume, Storage Conditions, Pre-Processing, Lysis Conditions and System Used to Capture/Elute RNA, for the Viral Load Assays

	Abbott RealTime HIV-1 (m2000rt)	COBAS® AmpliPrep/COBAS® TaqMan HIV-1, v2.0 (Roche)	NucliSens® EasyQ HIV-1 v1.2 (bioMérieux)	VERSANT® HIV-1 RNA 1.0 (kPCR) (Siemens)	Artus HIVirus-1 QS-RGQ (Qiagen)
Valid samples	Human plasma with ACD solution[1] or EDTA, DBS[2]	Human plasma with EDTA, DBS	Human plasma with EDTA, DBS, tissues	Human plasma with ACD solution[1] or EDTA, DBS	Human plasma with EDTA
Sample volume	Optimum amount 1.0 ml but uses 0.6 ml.	Optimum amount 1.0 ml but uses 0.85 ml.	0.5-1.0 ml	0.7-1.35 ml depending on the type and size of tube	1.2 ml
Pre-processing storage conditions	Whole blood: Room Temp. 24 h/2-8ºC 24 h Plasma: Room Temp. 24 h/2-8ºC 5 days/-80ºC indefinitely Freeze/thaw once only	Whole blood: Room Temp. 24 h 6ºC Centrifugation: 20 min Plasma: Room Temp. 24 h/2-8ºC 5 days/-80ºC indefinitely Freeze/thaw up to five times	Whole blood: Room Temp. 4 h Plasma: 48 h 2-8ºC/-80ºC indefinitely Freeze/thaw up to three times	Whole blood: Room Temp. 6 h, 2-8ºC 24 h Plasma: 5 days 2-8ºC/-80ºC indefinitely Freeze/thaw up to four times	Whole blood: Room Temp. 6 h Centrifugation: 20 min Plasma: 4ºC several days/-20ºC several weeks/-70ºC months and years
Sample preparation prior to lysis	Clarify by centrifugation at 2000 g x 5 min, uncap; place in the rack with the code facing outwards	Insert the clips with the codes in the rack, add the K-tubes, vortex the samples 3-5 s, pipette the samples into the tubes, place them in the rack	Pipette the lysis buffer into the apparatus, pipette the samples into the tubes, mix and add the mixture of silica and internal control	Place the samples in the tube rack with the code facing outwards and at the appropriate height	Prepare the internal control-RNA carrier mixture, place the samples in the tube rack with the code facing outwards
Lysis	Sodium guanidine isothiocyanate	Incubate with protease and lysis with guanidine thiocyanate	Sodium guanidine isothiocyanate/high-concentration saline	Proteinase K and chaotropic buffer	Proteinase K
Capture/elution of RNA	Magnetic particles/water	Capture technique based on generic silica/elution with aqueous solution at high temp.	Magnetic silica/water	Magnetic silica	Magnetic particles

[1]ACD- citric acid-citrate-dextrose.
[2]DBS: Dried blood spots.

interpretation systems. The report typically gives a classification into “susceptible", "possibly resistant" or "resistant" for each antiretroviral agent. Interpretation rules and algorithms can vary greatly from one to another [20]. Currently the protease (Pro), reverse transcriptase (RT), integrase (INT) and the V3 loop are the regions of interest for HIV-1 DNA sequencing. Pro, RT and INT are used to investigate resistance to protease, reverse transcriptase (nucleoside/otide and non-nucleoside analogues) and integrase inhibitors, while V3 sequencing is used for co-receptor usage estimation (tropism testing) prior to the use of CCR5 antagonists.

The most widely-used tests in routine clinical laboratories are those investigating RT and Pro mutations. Two commercially available FDA-approved and CE-marked kits are available: TRUGENE HIV-1 Genotyping Assay (Siemens NAD) and ViroSeq® HIV-1 Genotyping System (Celera-Abbott diagnostics). Although there are some differences in performance, both tests are labour intensive, both for the sequence generation and detection steps. Both methods generally provide concordant results in the way they detect mutations, but they differ in the way resistance reports are given [21].

Compared to phenotyping, the advantages of genotyping tests include less complexity, shorter turn-around time and lower cost, while their main limitations are: being an indirect measure of resistance; that they only interrogate known mutations; the difficulty in interpretation when complex patterns of mutations are present; the differential effect on resistance that combinations of individual mutations may have compared to individual mutations alone; and that they cannot detect low-abundance resistant mutations (minor variants).

Table **8** shows the main features of two tests, including technical, complexity, biosafety and cost-effectiveness aspects.

Minority Variant Detection and Tropism Testing

Conventional resistance tests can detect mutations which comprise over 20% of all the viral genomes amplified with RT-PCR. We are now at the stage where it is becoming important to detect mutations that might be between 1% and 20%, since it has been demonstrated that these patients are at greatest risk of failing therapy [22]. Techniques are therefore being introduced which are capable of detecting and/or quantifying mutation levels below the 20% threshold imposed by the currently-available techniques.

One of the methods already available, allele-specific real-time PCR (AS-PCR), can detect mutations present in as low as 0.01% of the population as a whole [23]. Despite this extraordinary sensitivity, however, the great disadvantage of this method is that an assay for every allele of every mutation is needed. This has meant that the only results presented have been for detecting the main mutations

Table 6. Ease of Use and Characteristics Relating to Instrumentation, Extent to which they are Automated, Use of Primary Tubes, Pipetting Steps and Connectivity with the LIS, of Five Commercially Available Viral Load Assays

	Abbott RealTime HIV-1 (m2000rt)	**COBAS® AmpliPrep/COBAS® TaqMan HIV-1, v2.0 (Roche)**	**NucliSens® EasyQ HIV-1 v1.2 (bioMérieux)**	**VERSANT® HIV-1 RNA 1.0 (kPCR) (Siemens)**	**Artus HIVirus-1 QS-RGQ (Qiagen)**
Instrumentation	m2000sp + integrated computer and m2000rt + integrated computer	COBAS Ampliprep + TaqMan 48 + computer	EasyMAG (extraction) + computer and EasyQ + computer	Sample extraction module (SP module) + amplification and detection module (AD module)	QIAsymphony SP/AS + Rotor-Gene Q + integrated computer
Ease of use	Fully automated, very easy to load. Automated extraction equipment separate from amplification/detection equipment	Simple to use, very easy to load. Equipment integrated onto one platform	Easy to use. Automated extraction equipment separate from amplification/detection equipment, manual pipetting of master mix	Fully automated. Extraction equipment separate from amplification/detection equipment	Automated extraction equipment separate from amplification/detection equipment
Reading of primary tube by barcode	Yes	No	Yes	Yes	Yes
Pipetting steps	Add the internal control to the extraction buffer prior to loading the reagents	Pipetting of the samples into the Roche equipment tubes	Additional pipetting steps during the extraction and amplification	Not required	Preparation of the internal control-RNA carrier mixture
Connection to LIS[2]	Yes, bidirectional	Yes, bidirectional	Yes, bidirectional	Yes, bidirectional	Yes, bidirectional

[2]LIS - laboratory information system.

Table 7. Efficiency of Viral Load Assays in Terms of Workflow (Hands-On Time Before Loading the Instrument, Number of Tests Per Run, Time Needed for Extraction and Detection and Time to Results)

	Abbott RealTime HIV-1 (m2000rt)	**COBAS® AmpliPrep/COBAS® TaqMan HIV-1, v2.0 (Roche)**	**NucliSens® EasyQ HIV-1 v1.2 (bioMérieux)**	**VERSANT® HIV-1 RNA 1.0 (kPCR) (Siemens)**	**Artus HIVirus-1 QS-RGQ (Qiagen)**
Preparation time prior to loading the samples for extraction	30 min	30 min	45 min	15 min	30 min
Number of tests/run	96	72	72	96	72
Extraction time	2.5 h/48 tests	2 h/24 tests	40 min/24 tests	2.5 h	1.5 h/24 tests
Detection time	3 h	3.75 h	1 h	2.5 h	3 h
Work flow and time for results	6 h	6 h	4 h	5.15 h	5 h

associated with resistance to non-nucleoside analogues (K103N, Y181C, G190A) and M184V [23-25], a mutation that has been associated with resistance to the main nucleoside analogues.

This drawback is overcome by the massively ultra deep parallel sequencing techniques (UDPS) which have been developed in the last few years and are able to detect mutations from 0.5%. Moreover, as these are single genome sequencing techniques, they can provide data on the percentage of each mutation compared to the whole population and on the mutational load, which is proving to be extremely useful as a true parameter in the interpretation of resistances. Another advantage of these platforms is that by sequencing single genomes, we are not limited to the known mutations, but with the introduction of the new antiretrovirals, we will be able to read each of the potential positions of interest in the future.

There are currently various UDPS platforms available. Table **9** provides a summary of the different characteristics of the platforms which are more advanced in terms of development, highlighting two principal aspects. The first is the length of the readings generated which, with the Roche platform (454) [26, 27], is close to that obtained by the Sanger method, making them ideal for single genome sequencing, although the great disadvantage is that they provide poor resolution in homopolymeric sequences. In contrast, Solexa [28] and SOLiD [29] have the great advantage over pyrosequencing of providing good resolution of the homopolymeric regions, but are not capable of generating readings beyond 75 bases and they cannot be used for *de novo* sequencing. Another important aspect is that they are able to sequence the DNA without it first having to be cloned, saving a lot of work and laboratory space. With these new sequencers, the cost of each nucleotide has fallen from $10 in 1990 to $0.01 in 2012.

Table 8. Technical, Complexity, Biosafety and Cost-Effectiveness Aspects Related to Commercial Tests Available for Resistance Testing

	ViroSeq™	TRUGENE®
US FDA approved CE marked	Yes	Yes
HIV-1 subtype	FDA approved for subtype B, but may also work for non-B	FDA approved for subtype B, but may also work for non-B
Viral load	>2.000 copies/ml, but may also work for 500-1000 copies/ml.	>500-1000 copies/ml.
Plasma Volume	≥0.5 ml	≥0.2 ml
Coverage	Pro: positions 1 to 99 RT: positions 1 to 335	Pro: positions 4 to 99 RT: positions 38 to 248
Cross-contamination control	UNG system (destroys PCR amplicons containing dUTP)	Built-in Genetic Fingerprint for sequence analysis
Labour intensiveness	Moderately high	High
Sequence data analysis	Experience needed	Semi-automatic
Instrument Maintenance cost	Expensive	Moderate
Sequence data analysis software	System specific	System specific
Resistance Report	Yes	Yes
Workflow	PCR& Sequencing purification steps 3 days for results	No purification 2 days for results
Biosafety requirement	BSL II cabinet	BSL II cabinet and specific chemical disposal requirement
Biohazard waste generated	Moderate	High, including specific requirement for handling chemical waste
Labour intensiveness (sequence detection)	Moderate, batch run	High, individual patient sequence run
Cost-effectiveness consideration for use	Surveillance and medium-to-large population-based genotyping services	Small-to-medium patient genotyping services
No. tests/kit	48	30

Table 9. Main Characteristics of the Platforms Available for Ultra Deep Parallel Sequencing (UDPS)

	GS-FLX (454)/ GSJunior	SOLEXA	ABI SOLID
Company	Roche	Illumina	Applied Byosystems
Sequencing method	Polymerase (pyrosequencing)	Polymerase (reversible terminators)	Ligase (octamers with two-base encoding)
PCR technique	Emulsion PCR	Bridge PCR	Emulsion PCR
Read Length (pb)	250-400	35-75	25-75
Run time (h)	10	48	168
nt/run (Gb)	0.4	18	30
Advantages	Amplicon size	Cheap	Cheap
Disadvantages	Homopolymers and manual	No *de novo* and manual	No *de novo* and manual

The great disadvantage of these techniques is that they are not automated. Although systems are being developed to enable the different steps to be automated, in the meantime, it has to be said that they are very laborious manual techniques, they take up a lot of time and require highly qualified personnel. So far, these platforms are not being routinely used in the laboratory. However, we are sure that in the not-too-distant future, that will change and these ultra-sequencing techniques will be available for more general use.

With regard to tropism testing, tropism can be inferred using phenotyping methods (Antivirogram® (Virco) [30], Pheno Sense® (Virologic) [31], Phenoscript® (VIRAlliance) [32] and MT-2 assay [33]), which are only possible in specialised laboratories or private companies that provide the logistics for collection and transport of samples. It can also be determined by using a genotyping method consisting of sequencing the V3 region of the gp140 envelope gene; depending on the sequence the quasi HIV species present, the virus will have R5 tropism (use the co-receptor CCR5) or X4 tropism (use the co-receptor CXCR4). The interest in this determination is that, before being

able to use a CCR5 antagonist (the only one approved so far is Maraviroc) it has to be confirmed that the patients are carriers of virus with R5 tropism. At present, there are no commercially-available tests with CE marking or FDA approval for determining viral tropism. These techniques have not been automated in the laboratory and manual procedures have to be used in order to obtain results [34].

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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Declared none.

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