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REVIEW ARTICLE

ASSASYING THE NEED OF COMMERCIAL PLASMA VIRAL LOAD TESTING IN RESOURCE LIMITED SETTINGS

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ABSTRACT: Around nine million Human Immunodeficiency Virus (HIV) infected individuals are on antiretroviral therapy (ART). People living with HIV/AIDS in resource-limited settings where HIV burden is usually high, there is an urgent need of affordable, accessible and inexpensive tests to monitor response to treatment. Quite a few commercially available assay has been introduced to measure Plasma Viral Load (PVL) as testing can increase adherence to ART and facilitate timely switching of failing regimens and thus minimizing the development of resistance. We analyzed Nucleic Acid Test (NAT) based assay and Non Nucleic Acid Test based assay for PVL testing. Though both the assay has its own advantage and disadvantages, but the use of Non Nucleic Acid Test has an upper hand in resource limited settings. It is the duty of administration, clinicians, microbiologist and health care personnel to introduce appropriate laboratory monitoring assays in resource-limited settings.

KEYWORDS: Plasma Viral Load, Nucleic Acid test, Non Nucleic Acid Test, HIV, Resource Limited Settings.

INTRODUCTION: It is estimated worldwide that over nine million Human Immunodeficiency Virus (HIV) infected individuals are on antiretroviral therapy (ART) and a substantial proportion have been on treatment for years.[1] Improved access to ART through price reductions, availability of drugs, and significant increases in funding from all corners of world have resulted in a dramatic expansion of the number of HIV infected individuals receiving treatment in low and middle-income countries.[2] People living with HIV/AIDS in resource-limited settings where HIV burden is usually high, there is an urgent need of affordable, accessible and inexpensive tests to monitor response to treatment.[3] A lot of simplified public health approach that has been used to provide large-scale drug delivery; has been found in limited studies to be cost effective.[4] Though HIV treatment is manageable without routine laboratory assessment,[5] a CD4+ T cell count monitoring helps and allows the evaluation of disease progression[6], and Plasma Viral load (PVL) testing can increase adherence to ART and facilitate timely switching of failing regimens and thus minimizing the development of resistance.[7,8] More recently the focus has shifted toward evaluating the possibility and access to laboratory assays, such as measurement of CD4+cell count and PVL, for these regions to support HIV clinical management programs. One of these laboratory tools, the PVL assay, has become the standard of care for monitoring patients receiving ART in the developed world.[9,10] The argument rose by Badri et al that monitoring of CD4+ cell count is an inadequate alternative to monitoring of viral load and cannot be used to substitute for viral load monitoring is receiving steadily growing support.[4] World Health Organization (WHO) guidelines published in 2004 for ART in resource-poor environments suggested that CD 4+ cell count measurement was desirable for initiation of treatment but that the use of viral load for monitoring was optional. These guidelines have been revised to suggest that increased access

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to virological testing is highly desirable, particularly for clinical decision making related to switching drug regimens[11]. Monitoring HIV PVL is often not performed in resource-limited settings because the assays are costly, and require sophisticated, expensive laboratory equipment and trained technicians.[12,13] Globally ART cohort continues to expand and mature, hence the need for ongoing monitoring is becoming increasingly important to ensure treatment efficacy and minimize the risk of drug resistance. A number of nucleic acid test (NAT) based assays has been developed that use reverse transcriptase polymerase chain reaction (RT-PCR), branched DNA (b-DNA), and nucleic acid sequence based amplification (NASBA) technologies.[14] Nucleic acid–based amplification assays are the mainstay of viral load monitoring in high-income countries. Assay design is complicated by the high level of genetic heterogeneity characteristic of HIV-1 and the emergence of recombinant strains.[15]

Nucleic Acid Amplification Assays:

Nucleic acid amplification tests are divided into two parts:

i. Target amplification.
ii. Signal amplification.

Detection is done using either end point polymerase chain reaction (PCR) or real-time PCR. The advantages of nucleic acid testing approaches are that many of these assays have been well validated and are available in quality-assured kits, and there is clinician familiarity with interpretation of results. Most of these assays vary considerably by sample preparation, amplification and detection methodology, region of the genome targeted, and dynamic range.[16]

Nucleic Acid Tests by some diagnostic companies;

1. Roche (Roche Amplicor and COBAS Taqman);
   a. Roche Amplicor Monitor (version 1.5), which uses 3 different formats (manual, manual extraction, and comprehensive bioanalytical system.
   b. COBAS amplification and detection; COBAS Ampliprep/COBAS Amplicor (Roche Molecular Systems).[18-20]
2. Bio Merieux (NASBA) NucliSENS EasyQ (versions 1.2 and 2) - The NucliSENS assay is a nucleic acid sequence–based amplification methodology using a molecular beacon detection format that can be semi-automated with use of the manual NucliSENS miniMag extraction methodology or automated using the EasyMag analyser.[19-21]
3. Abbott (Real time) (b-DNA) - Abbott HIV-1 RealTime assay (Abbott Diagnostics) have developed HIV-1 viral load assays. The Roche (version 1.5) assay is a reverse-transcription PCR– based, target amplification assay targeting the gag p24 region of the genome.
4. Siemens (bDNA) Versant HIV-1 RNA assay (Version 3.0; bDNA; Siemens) is a nucleic acid hybridization method that depends on signal amplification performed in a 96-well format on the 440 bDNA analyser.
5. Biocentric generic viral load assay - A real-time PCR assay called the Biocentric generic viral load assay is available from Biocentric that can be placed on a variety of real-time analysers and is referred to as an open system.[16]

In comparison to previous end-point detection methodologies, few companies have developed the real time technologies which are based on fluorescence to achieve simultaneous amplification

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and detection instead of end-point detection.[17] These assays extend the subtype coverage to detect increasing numbers of non-B strains, which are prevalent in most affected countries. However, most of NAT PVL assays are expensive for the use in resource-crunched settings and require very sophisticated infrastructure facilities. Monitoring PVL in resource-limited settings faces tremendous challenges particularly;

i. Lack of bare minimum facility like electricity, water and air condition to perform the test
ii. Lab personnel lacking knowledge and skill of molecular biology to run the test
iii. Improper financial support with lack of transparency in the funds
iv. Not being robust methods.

Assay	Bio-merieux		Siemens	
References	**33-35**	**36-37, 51**	**38**	**39-40**
Variable	NucliSENS HIV-1QT	NucliSENS EasyQ HIV-1 (version 2.0)	VERSANT HIV-1 Quantiplex (version 3.0; bDNA)	VERSANT HIV RNA (version 1.0; kPCR)
Assay type	NASBA; isothermal amplification; electro chemiluminescence detection; manual extraction, NucliSENS extractor, or Nuclisens miniMAG	NASBA; real-time detection; molecular beacons; Nuclisens miniMAG (Automatic); Nuclisens EasyMag (Manual)	bDNA; sandwich nucleic acid hybridization method; signal amplification	Real-time PCR
Linear range, RNA copies/ml	51–5 million	10-10,000,000	50–500,000	30-11,000,000
Specimen type	Plasma, serum, DBS, any body fluid, EDTA, citrate, heparin	Plasma, serum, DBS, any body fluid	Plasma, EDTA, ACD	Plasma, serum, DBS, EDTA
Specimen volume µl	200–1000	200–1000	1000	500
Area of genome targeted	Gag; target amplification	Gag; signal amplification	Pol; target amplification	Pol; target amplification
Controls	3 Internal calibrators (synthetic RNAs): Qa (high), Qb (medium) and, Qc (low); positive, negative controls not supplied with kits and left to lab decision	1 Internal calibrator; no external controls provided and inclusion reduces throughput	6–9 Standards and 3 controls (negative, low, high) per plate	Positive (high, low); negative
Subtype reactivity	Group M; not suitable for G, O	Group M; (A-D, F-H, J) not suitable for O, G	Group M	Group M (clades A–H, CRF01-AE, CRF01-AG and CRF06-cpx); Group O

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Technical Skills	High	High-Med	High-Med	High
Lab Set Up	Centrifuge/vortex; water bath/heating block; biohazard hoods; reader	Centrifuge/vortex; analyzer; biohazard hoods	Siemens 340 or 440 molecular system; biohazard hoods, refrigerated centrifuge, heating block, water bath, vacuum system	Main system VERSANT kPCR molecular system
Thorough Output (no of samples/run	20–30	miniMAG (12 per run); EasyMAG; EasyQ analyzer (48 per run)	12–168	89 (+7 calibrators and controls)
Time to result in hrs	3.5	4	22	6
Cost/ kit	40–100	40–60	125	30–75
FDA Approval	Yes	No	Yes	No
CE Marketing	Yes	Yes	YES	Yes
Advantage/s	Isothermal; many sample types	Closed system; rapid; automated; medium technical skill	High throughput; Versant 440 system fully automated; no special laboratory set-up required; no separate extraction or amplification areas	Real time; can be fully automated; amerase to prevent contamination
Disadvantage /s	Contamination risk; postamplification steps required; dedicated space and equipment; high technical skill; cost; technical support required	Dedicated space and equipment; cost; technical support required; not FDA approved; significant risk of contamination in high-volume laboratories	Dedicated space and equipment; technical support required	Dedicated space and equipment; high technical skill; cost; technical support required

Table 1 Contd....

Assay	**Roche Molecular Systems**		**Abbott**	**Biocentric**
References	38, 52	38, 41-44, 53	38, 45-47, 54	48-50
Variable	Amplicor HIV-1 Monitor (version 1.5)	COBAS Taqman	RealTime HIV-1	Biocentric Generic viral load
Assay type	RT-PCR; end point PCR; Microwell Plate Manual and COBAS Amplicor or COBAS	RT-PCR; real-time PCR; dual-labeled hydrolysis type probes; Armored RNA internal	RT-PCR; real-time PCR; partially ds real-time probe with fluorescent label; armoured	Real-time PCR; RT

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	Ampliprep/Amplicor	quantitation standard (HIV QS); Two versions (version 1 and 2)	RNA internal standard; automated via m2000rt; automated extraction (Abbott m2000sp, previously m1000 Abbott LCx assay largely discontinued	
Linear range, RNA copies/ml	400–750,000 (standard); 50–100,000 (ultrasensitive)	40–10 million for version 1; 20–10 million for version 2	40–10 million	300–50 million
Specimen type	Plasma, Dried Blood Spot (DBS)	Plasma, DBS	Plasma, DBS, EDTA, ACD	Plasma, DBS
Specimen vol.	200 or 500	1000	200-1000	200–500
Area of genome targeted	Gag; target amplification	Gag; target amplification; In version 2LTR and gag regions targeted	Pol integrase region; target amplification	LTR; target amplification
Controls	High positive; low positive; negative; internal control	High positive; low positive; negative; internal control standard	Low positive; negative; internal control	One internal calibrator; no external controls provided
Subtype detecivity	Group M; not suitable for O	Group M (A-D, F-H; CRF01_AE) group O included for version 2.0	All; better for CRF02-AG recombinants	Group M; not suitable for O, G
Technical Skills	High-Med, if automated	High-Med, if automated	High-Med, if automated	High-Med
Lab Set Up	For manual, thermocycler, ELISA reader, washer, and microcentrifuge; for automated, COBAS Ampliprep and COBAS Amplicor analyzers	COBAS Taqman with or without COBAS Ampliprep 48 or 96 system; biohazard hoods; centrifuges; 168/8 h day per continuous loading	Automated extraction and prep (m2000sp); amplification and detection (m2000rt); bar code reader for primary tubes; centrifuge; biohazard hood	Thermocycler; biohazard hoods; Centrifuge
Thorough Output (no of samples /run)	12–48 (9–21 per run)	48 or 96	48 (includes 3 controls)	1–96 samples/run
Time to	7	5	5	4h

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result in hrs				
Cost/ kit	20-100	30–100	20–40	14
FDA Approval	Yes	Yes (for version 1)	Yes	No
CE Marketing	Yes	Yes (for version 1)	Yes	Yes
Advantage/s	Limited linear dynamic range; 2 versions; dedicated space and equipment; cost; technical support required	Amperase to prevent contamination; single tube; fully automated; Medium technical skill; wide dynamic range; Ampliprep can be docked to Taqman for automated sample transfer; single room required; Amplilink software for interfacing	Closed system post-PCR [41]; wide dynamic range; can be fully automated	Close system; rapid; automated; high technical skill; open system; difficult to quality control all components
Disadvantage /s	Limited linear dynamic range; 2 versions; dedicated space and equipment; cost; technical support required	Dedicated space and equipment; may have docking of COBAS Ampliprep/Taqman allowing 1 room; cost; technical support required	Dedicated space and equipment; cost; technical support required	Dedicated space and equipment; high technical skill; capital equipment expensive; technical support required; not FDA approved

Table 1: Comparative Analysis of Various Nucleic Acid Test Based Assay[16]

Non Nucleic Acid Testing: The high cost of NAT test along with high cost of reagents and consumables restricts its implementation in recourse limited settings. In order to assay PVL monitoring in resource limited settings, alternative accessible inexpensive methods have been developed by different manufacturers and some of these have been validated in the recent years 16 such as the Cavidi ExaVir™ RT assay,[22-24] and PerkinElmer Ultrasensitive p24 assay,[23, 25, 26] are based on ELISA method, measuring the activity of reverse transcriptase (RT) enzyme and the concentration of p24 antigen. The use of these indirect measures of PVL require less equipment and skill , have thus been evaluated for resource-poor settings and include the ultrasensitive, heat-denatured p24 antigen quantification assay which is no longer being developed for viral load monitoring, and the ExaVir Load (Cavidi AB) reverse-transcriptase assay[13,27,28]. Recently, other approaches have included the evaluation of flow cytometry–based markers of activation, such as the quantification of bright CD38 expression on CD8 cells[29, 30.]

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Variable	Ultrasensitive, heat-denatured p24 antigen quantitation assay (Perkin Elmer Life Sciences)	ExaVir load (version 3; Cavidi)
References	56	28, 55,56
Assay type	Enzyme immunoassay for quantitation of p24 antigen; separate external buffer required	Enzyme immunoassay for quantitation of reverse transcriptase activity; colorimetric reading
Detection	Colorimetric or fluorimetric; Quanti kin detection software	Colorimetric; detection software provided by company with kit start up package
Dynamic range, RNA copies/ml	Clinical cutoff: 10,000–30,000 RNA copies/mL; reported as fg/mL: 1–3000fg/mL	RNA copies/mL equivalents; version 3: 200 to 1600,000 RNA copies/mL equivalents
Specimen type	Plasma (ACD, CDPA-1, EDTA, sodium citrate, or heparin), serum, or cell culture supernatant	Plasma
Specimen volume	200–450	1-1000
Controls	5 Concentrations (VQA serum quality controls and diluents correction controls not provided in kit)	Serially diluted standard and recommended plasma pool for control
Subtype reactivity	HIV-1 (all subtypes); HIV-2	HIV-1
Time to result	2.5 h	2.5 days (colorimetric); 1.5 days (fluorimetric)
Technical Skills	Low to medium	Low to medium
Lab Set Up	Simple ELISA plate washer and reader; incubator; refrigerator and/ or freezer; dry heat block	Incubator; freezer to store samples; ELISA plate washer and reader
Thorough Output (no of samples/run	High; 96 samples per run	32 samples per run
Cost/ kit	$20–$30	$15–$25
FDA Approval	No	No
CE Marketing	No	Yes
Advantage/s	Easy training; simple ELISA plate washer and reader; no separate areas required; high throughput; can be used for infant diagnosis of HIV infection [27]	Easy training; simple ELISA plate washer and reader; no separate areas required; can be used for NNRTI resistance monitoring
Disadvantage/s	Not sensitive enough to facilitate treatment changes in any setting; inconsistent supply; external buffer required	Controls not supplied

Table 2: Comparative Analysis of Various Non-Nucleic Acid Test Based Assay[16]

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Advantages	Disadvantages
It is simple to perform	Lengthy and tedious process and takes time approx of 72hrs
It is inexpensive	1 ml of plasma is required, which is difficult to get in pediatric patients
It does not require sophisticated infrastructure facilities and expensive equipment	a lack of standard positive and negative controls [34]
No requirement of highly skilled technical staff	Requires skilled staff

Table 3: Advantages and Disadvantages Cavidi Exavir™ PVL Assay over the Standard RT-PCR Assay are,

Though the alternative methods (Non NAT methods) have dramatically reduced the cost $15-20 in comparison with NAT based PVL assay which costs $ 70-100.[16] Both the assay described here viz ExaVir™ assay and the ultrasensitive p24 assay are much simpler and cost effective than all the NAT methods.

Proposed Viral Load Testing Algorithm for Resource-Poor Environments: It is recommended in high income countries that viral load testing be conducted at baseline; 2–8 weeks after initiation of ART to assess early virological response and then after every 3 -4 months[32]. This may not be in case of resource limited countries. Non-NAT are appropriate in such cases to measure PVL. The quality of testing is an important consideration in the selection of technologies. Even if the best test is purchased, if the test is performed badly, the results will not be useful. The selection of a VL technology needs to take into account the following;

1. Can staff be trained on the technology with ease?
2. Is there access to technical support?
3. Is it possible to perform regular maintenance?

CONCLUSION: Not a single stone should be left unturned to develop and introduce appropriate monitoring assay in resource-limited settings, as the benefit of ART with minimum drug resistance seems to be largely dependent on adequate monitoring. Issues like cost factor, adequate laboratory monitoring, skills and knowledge of technical staff, self-life of kits and reagent and there delivery should be taken into consideration apart from External quality assurance schemes (EQAS) should be taken into consideration at the time of implementation. The assays should be validated to ensure that the results are reproducible reliable and accurate.

The NAT test and Non NAT test works on two different principles. The NAT or Plasma HIV RNA assay measures virion-associated RNA while the Non NAT or RT assay measures the activity of a virion-associated enzyme required for replication. Measuring RT enzyme activity as marker for PVL has several advantages in the international setting apart from being inexpensive with less technical skills.

The reverse transcriptase enzyme is well conserved across different genetic clade types; therefore the RT assay would be expected to function well in areas of the world where non-clade B

strains, including complex recombinant strains, are common. While the NAT assay cannot differentiate between functional and defective virions and therefore may not reflect true viral replication activity, whereas the RT assay quantifies actual reverse transcriptase activity.[44] It is the duty of administration, clinicians, microbiologist and health care personnel to introduce appropriate laboratory monitoring assays in resource-limited settings. As we are highly burdened by HIV/AIDS all effort should be counted in providing better and cheaper method for diagnosis and prognosis of HIV/AIDS as PVL has emerged as an important factor.

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