

Quantitative nucleic acid amplification methods and their implications in clinical virology

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ABSTRACT

Recently, a number of techniques have been approved for quantification of viral nucleic acids in clinical samples. Viral load (VL) tests have considerable importance in the management of patients and are widely used in routine diagnosis. In clinical virology, VL testing are important to monitor the antiviral treatment, to initiate preemptive therapy, to understand pathogenesis, and to evaluate the infectivity. These tests have now become a part of many diagnostic and treatment guidelines. Considering the various challenges for in-house viral testing related to the standardization, validation, and precision; they are gradually being replaced by the United States Food and Drug Administration (US FDA) cleared tests. This review summarizes the various viral quantification methods and also discusses the clinical applicability of these in human immunodeficiency virus, Hepatitis B virus, Hepatitis C virus, Cytomegalovirus, and Epstein Barr virus infected patients. Further the challenges and future perspectives of VL testing have also been discussed.

Key words: Diagnosis, monitoring, preemptive therapy, viral diseases, viral load

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INTRODUCTION

The nucleic acid amplification techniques have played an important role in diagnostics over the years. The initial qualitative techniques have now by enlarge been replaced by quantitative methods which are effective in many applications in medicine. Recently, virus quantification has been used as a direct method of measuring replicating virus and various VL assays play an important role in patient management. These tests can be used to monitor the efficacy of therapy, to identify the emergence of drug resistance, to make decisions to initiate preemptive treatment, to assess disease progression and also for the diagnosis.

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VIRAL QUANTIFICATION TECHNIQUES

VL is usually expressed as the number of nucleic acid copies per milliliter of blood or in terms of International Units per milliliter. In human immunodeficiency virus (HIV), it is typically reported as copy numbers^[1] while hepatitis B virus (HBV) DNA and hepatitis C virus (HCV) are usually expressed in International Units per milliliter to ensure comparability. The changes in VL are usually reported as a log change (in powers of 10). Depending on the commercial kit used and its respective conversion factor, the values given as copies per milliliter can be converted to International Units per milliliter.^[2]

The VL quantification methods can be divided into target-, signal-, and probe-based amplification methods.

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TARGET-BASED METHODS

Polymerase chain reaction

The most commonly used quantification technique is the real-time polymerase chain reaction (PCR) which can quantify in the following two ways:

- i. Relative quantification: In this method, the amplification efficiencies of targets are normalized with respect to reference gene and then subjected to quantification. It has a limited role in clinical practice.^[3,4]
- ii. Absolute quantification: This method requires the preparation of standards curves which can be done using DNA standards with known concentration/or recombinant plasmid containing the target.^[5,6]

Nucleic acid sequence-based amplification

It can be used for the continuous amplification of nucleic acids in a single mixture at one temperature given. This has been used for various viral diseases such as HIV, HCV, norovirus and chikungunya.^[7-10]

Transcript mediated amplification

It is amplification method which uses both RNA polymerase and reverse transcriptase for the amplification of target molecules which can be RNA/DNA. It has good sensitivity for the detection of HCV and has also been used in conjunction with branched DNA for quantitative testing of HCV.^[11,12]

Loop-mediated isothermal amplification

It allows isothermal amplification of target gene and utilizes 6 primers sets for loop formation. It is a rapid, specific, and cost-effective method for diagnosis which can be carried out even in a field setting.^[13]

Digital polymerase chain reaction

It is an advanced form of quantitative PCR (qPCR) which can detect and quantify the low level of viruses. As compared to Ct values in real-time PCR it gives a signal which decreases its variability. It directly measures the amount of DNA (absolute quantification) without preparation of standard control.^[14,15]

SIGNAL-BASED AMPLIFICATION METHODS

Branched chain amplification: Branch DNA assays

In these techniques, the target viral nucleic acid is captured onto the solid phase by oligonucleotide probes. The combination of synthetic oligonucleotide probes measures the amount of nucleic acid. This technique has high sensitivity and reproducibility and is the basis of a Food and Drug Administration (FDA) approved test for HIV.^[16]

Hybrid capture

This technique detects the DNA by the formation of DNA-RNA hybrid using RNA probes. The DNA-RNA hybrids are then captured by antibodies, and the signal is measured in the form of relative light unit. The technique is highly used for the monitoring of human papillomavirus (HPV) load in various risk groups by using digene Hybrid Capture 2 test (FDA approved). Furthermore, it has been used for the detecting cytomegalovirus (CMV) load in transplant patients^[17] and for quantification of HBV DNA.^[18]

PROBE-BASED AMPLIFICATION METHODS

Ligase chain reaction

It amplifies the nucleic acid used as the probe for each of the two DNA strands; two partial probes are ligated to form the actual one, and require both polymerase and ligase for reaction. It is highly sensitive and specific test and can distinguish single base change, hence specifically used for detection of mutations rather than quantification *per se*.^[19,20]

Invader assay

The basis of this assay lies on the cleavage of structure formed from primary and invader probe. This technique has been used for the quantitation of closed covalently circular (ccc) HBV DNA and HPV 16.^[21,22]

ROLE OF VIRAL LOAD IN CLINICAL PRACTICE

The various clinical situations where VL detection can be useful are as a diagnostic marker, for therapeutic monitoring, for initiation of prophylactic therapy/preemptive therapy, study disease pathogenesis and for the estimation of infectivity.

Viral load as a diagnostic marker

Real-time PCR is important for the diagnosis of acute infections like HIV to document viremia in seronegative individuals [Figure 1]. The HIV-1 VL tests have featured in the recent HIV testing algorithm proposed by the Centre for Disease Control (CDC).^[23] Its role is also important in HCV and HBV in seropositive individuals to demonstrate viremia in the baseline samples. The role of VL is important in the case of latent viral infections to differentiate it from an active infection as in case of Epstein–Barr virus (EBV) and CMV infection where the infection is ubiquitous.^[24] In case of EBV infection, patients with symptomatic chronic active infection show higher copy number in blood (1.45×10^5 copies/ml) as compared to infectious mononucleosis patients (3.08×10^3 copies/ml), EBV-associated hemophagocytosis (2.95×10^4 copies/ml), or healthy controls ($<2 \times 10^2$ copies/ml).^[25] Its role in CMV is

important to differentiate between active and asymptomatic infection wherein the absolute VL will be high, or a rising trend would be seen in the case of actively replicating virus in contrast to those having latent infection.^[24]

In case of HBV infection, a patient is said to be in HBeAg-positive immune active phase if the VL is $\geq 2 \times 10^4$ IU/ml, HBeAg-negative immune reactivation phase if VL is $\geq 2 \times 10^3$ IU/ml and inactive chronic hepatitis B phase if VL is $< 2 \times 10^3$ IU/ml.^[26]

Viral load for therapeutic monitoring

VL testing has been used as a therapeutic marker to monitor the course of treatment, the decision to switch over therapy, monitor drug resistance and to predict the outcome of the present therapy. VL testing is indispensable for the monitoring of the following viral infections:

Human immunodeficiency virus

HIV-RNA load gives information on the degree of viral replication at the time of assay. It should be repeated every 3–4 months or as clinically indicated. For patients adherent to therapy, this interval can be extended to every 6 months.^[27] If the VL continues to be $> 5 \times 10^3$ copies/ml after 6 months of treatment, second line antiretroviral treatment is advised. In many cases, even after giving antiretroviral therapy (ART), viremia persists because of the presence of HIV in persistently and latently infected CD4 T-cells in the peripheral blood as well as gut-associated lymphoid tissue. This residual plasma viremia can be measured by the detection of HIV-proviral DNA, which may assess the release of infectious virions and also the number of infected cells. This, proviral DNA load can be used to detect early therapeutic failures and to follow-up the evolution of resistant viral variants under ART therapy,^[28] as well as a marker for the completion of therapy.

Hepatitis C virus

During HCV infection, VL is performed at the initiation of treatment, after 4 weeks of treatment (rapid virological

response [RVR]), 12 weeks after treatment (early virological response [EVR]) and at 24–48 weeks of treatment depending on the HCV genotype (end of treatment response, [ETR]). The undetectable HCV RNA after 24 cessation of treatment is defined by sustained virological response (SVR) [Figure 2]. The presence of RVR is a good predictor to attain SVR. The absence of EVR accurately predicts failure to achieve SVR and SVR is the best predictor of a long-term response to treatment. In this regards, two terms need mentioning: virological breakthrough which is the recurrence of HCV RNA in the patient who is yet on treatment and virological relapse which refers to the recurrence of HCV RNA in serum after the discontinuation of treatment and the documentation of an ETR. Null responders are the patients who fail to decline HCV RNA by < 2 logs after 24 weeks of treatment while the partial nonresponders are those patients in whom HCV RNA levels decline by ≤ 2 logs but are never undetectable.^[29] It has been seen that the patients with high pretreatment VL (6×10^5 IU/ml) or genotype 1 infections have lower SVR as compared with genotype 2 and 3 infections. Monitoring HCV RNA levels are important to determine the duration of treatment and as a guide to stopping treatment especially in lieu of the introduction of US, FDA approved newer protease inhibitors which have been shown to achieve SVR in 12 weeks in certain genotypes of HCV.^[30]

Hepatitis B

The measurement of HBV VL along with liver function test and other viral markers is used to make decision to start therapy.^[31] The patients should be considered for treatment when HBV DNA levels are more than $> 2 \times 10^3$ IU/ml and

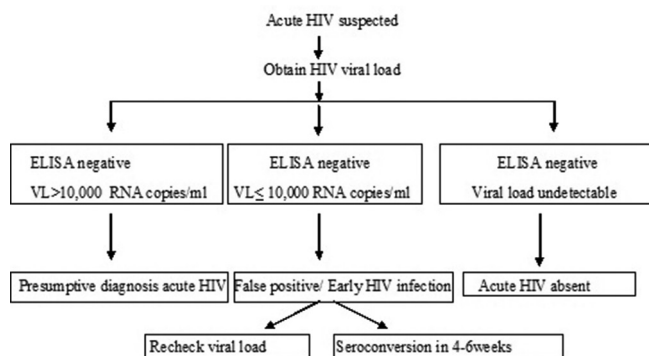


Figure 1: Algorithm for diagnosing acute human immunodeficiency virus

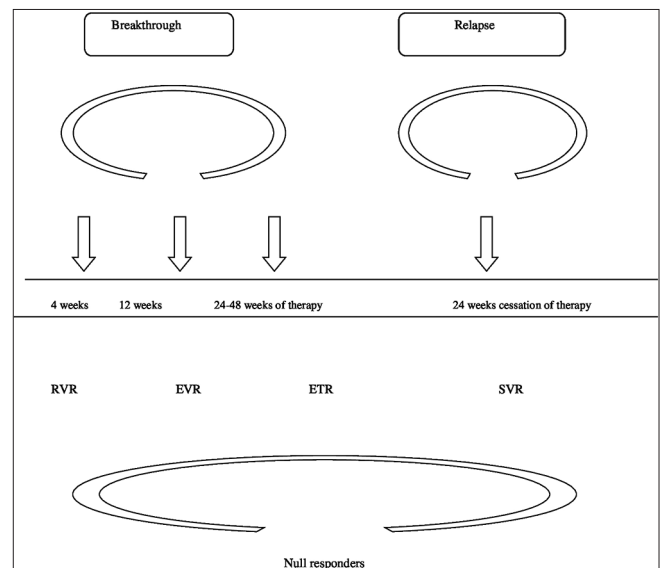


Figure 2: Timelines, at which viral load is monitored in hepatitis C virus infected patients, to evaluate the therapeutic response (breakthrough cases, relapses or null responders)

serum alanine aminotransferase (ALT) levels are high and there is moderate to severe active necroinflammation seen in liver biopsy with or without at least moderate fibrosis using standard scoring system.

In HBe Ag negative patients with normal transaminase levels, the ALT should be checked for 3 months, and the HBV VL should be monitored every 6–12 months for 3 years. VL markers are important to predict the emergence of drug resistance in patients on antiviral therapy since the treatment duration is long. The various terms which are important in this regards are virologic breakthrough, viral rebound, and virological failure.^[26]

Recent emphasis has now shifted from detecting HBV DNA to measuring the intrahepatic (IH) cccDNA (cccDNA), formed during HBV replication which leads to persistence of HBV infection. It has been shown that the IH cccDNA may persist in patients with acute hepatitis B and in patients with chronic hepatitis B who achieve virological response and hepatitis B surface antigen seroclearance following anti-viral treatment.^[32]

Cytomegalovirus

The measurement of CMV VL should be done at baseline and thereafter weekly when the patient is on treatment. CMV DNA load of $>10^3$ copies/ml after 2 weeks of ganciclovir treatment can be suggestive of drug resistance. The drug resistance can be seen in patients after solid organ transplantation, stem cell transplant patients, and HIV-infected patients.^[33,34]

Viral load as a guide to start prophylactic therapy/preemptive therapy

This is especially relevant in CMV and EBV virus infection. The monitoring of CMV VL is required in case of transplant patients (solid organ transplant [SOT] and hematopoietic stem cell transplant [HSCT]) weekly during the high-risk period, i.e., 12–14 weeks and 100 days posttransplant, respectively. In patients with allogeneic transplant, it is important to consider preemptive therapy with ganciclovir if CMV load is $>1 \times 10^4$ copies/ml. In hematopoietic cell transplantation, an initial VL of 1×10^3 copies/ml is considered an optimal threshold to start preemptive therapy.^[35] In SOT recipients, a VL of 1×10^3 – 5×10^3 copies/ml of plasma and in hematopoietic stem cell transplant recipients 400 copies/ml of plasma predict the likelihood of CMV disease. It has been seen that the solid organ transplant patients who had a VL of $<1 \times 10^4$ copies/ml had more than 2 fold higher chance of virus eradication at 49 days posttreatment compared to those with a VL of $>1 \times 10^4$ copies.^[24,36,37]

The VL testing has enabled the early diagnosis of posttransplant lymphoproliferative disease (PTLD) for monitoring the tumor burden over time. AVL of 10^3 genome equivalent/ml has been associated with EBV reactivation. The preemptive use of rituximab in transplant patients with EBV reactivation would prevent the development of PTLT, hence high-risk screening will be useful.^[38]

In case of BK virus infection, as per the Infectious Disease Society of America guidelines, preemptive monitoring in urine should be carried out 3 monthly and in plasma every 1–3 months up to 2 years posttransplant. As per the American Society of Transplantation, a BK VL of $>1 \times 10^4$ copies/ml for >3 weeks indicates presumptive BK virus nephropathy and immunosuppression reduction is advised.^[39,40]

Viral load to study disease pathogenesis

VL is a surrogate marker of persistence in certain viral infections and predicts risk of carcinoma in HPV, EBV, HBV, and HCV. In case of EBV, VL is an independent predictor of the risk of nasopharyngeal carcinoma (NPC).^[41] A higher VL has been documented in patients who had advanced stage NPC as compared to those with early-stage disease (4,70,47 vs. 5918 copies/ml).^[42]

Recent studies have suggested that HPV16 E6 and E2 VL can be a predictor of HPV infection. The VL detection in HPV has been based on HPV integration assays which measure the ratio of E2 and E6 genes. The E2/E6 ratio can be helpful in determining the physical status of HPV. The E2 gene disruption often results in integration of HPV. The ratio <1 is suggestive of viral integrated and episomal form. The various studies have suggested the good correlation of high-grade intra-epithelial lesions with high VL by using a qPCR for HPV 16 E6 and E2.^[43]

The presence of hepatitis B e antigen (HBeAg) and high HBV VL are independent risk factors for the progression to cirrhosis and HCC.^[26] The HCV patients who have achieved SVR but have cirrhosis are at a higher risk of developing HCC and death in short term and hence need periodic monitoring.^[29] Furthermore, hepatitis due to HBV reactivation is common in HBsAg positive patients undergoing autologous hematopoietic cell transplantation. A high prechemotherapy HBV DNA $>10^4$ copies/ml has been the most important risk factor for HBV reactivation.^[44]

Viral load for estimation of infectivity

Vertical transmission

In most of the cases of vertical transmission of HIV, infection occurs during delivery and the maternal

plasma VL strongly correlates with perinatal transmission. The chances of spreading are up to 80% in pregnant women if the plasma RNA load is $>10^5$ copies/ml while a load of $<10^3$ copies/ml is associated with little or no HIV transmission.^[45]

In case of hepatitis B, maternal HBV DNA $>1.5 \times 10^5$ is significantly associated with higher risk of intrauterine transmission.^[46] Similarly in case of HCV, viral titers of $>10^6$ – 10^7 copies/ml is associated with a higher risk of vertical transmission.^[47,48]

Horizontal transmission

The VL testing is important to assess the risk of transmission from a needle stick “donor” to the recipient. The Society of Healthcare Epidemiologist of America has recommended that HIV-infected doctors should wear double gloves for all the invasive procedures and they should not carry out category III activities such as general surgery, oral surgery, neurosurgery, transplant surgery, and trauma surgery associated with a risk for donor-to-recipient transmission of bloodborne pathogens if they have circulating HIV RNA of $\geq 5 \times 10^2$ copies/ml.^[49]

Similarly, the HBeAg-positive physicians should not carry out the “exposure-prone” procedures (EPP). However, HBeAg-negative health care workers also have occasionally transmitted HBV infection. The iatrogenic transmission of HBV can be prevented by estimating the plasma levels of HBV DNA.^[50] In Germany, the health care workers can practice if they have a VL of $<10^5$ – 10^6 HBV genome copies/ml.^[51] In the UK, threshold level of 10^3 copies/ml of plasma is taken into consideration for those indenting to carry out EPP.^[52] However, CDC recommends that a VL of $<1 \times 10^3$ IU/ml is safe for practice.^[7] In these regards, it is important to bear in mind that different assays may give different results which may not always be comparable.^[53] The risk of transmission from HCV-infected surgeons to patients is quite low but has also been frequently reported.^[54] Therefore, several national guidelines have recommended VL testing in HCV-infected health care workers.^[55]

CHALLENGES OF VIRAL LOAD TESTING

The accuracy of VL methods is questioned by the vast variability observed in quantitative results. Although the typical coefficients of variation in these tests should be in the range of 1–5%, sometimes it may go up to 30–40% or more; also wide fluctuations are seen among different laboratories.^[56,57] The process of virus quantification involves many sequential steps which may affect the test. The steps

involved in viral quantification may affect the test, hence to gain accuracy and reproducibility, standardization of each step is required. The use of various calibration methods can be helpful to overcome the variability in different laboratories.^[58] Also for VL testing (HIV and HCV), the availability of international standards, FDA approved kits, the use of calibration standards etc have attributed to improved inter- and intra-laboratory comparisons which has marked benefits for the patient management. In addition, the various laboratories should participate in National Quality Assurance Programme and National Accreditation Board for Testing and Calibration Laboratories for quality assurance purposes.^[59] Furthermore, the in-house testing should be replaced by use of FDA approved tests wherever available to ensure reliable reporting. This is especially important in case of HCV, HBV, and HIV where the baseline VL is required for treatment initiation, to see the duration and efficacy of antiviral treatment.^[60] Table I gives a list of the FDA approved tests for some of the common viral infections.

CONCLUSION

Although the molecular techniques are a boon to humankind, they are expensive. There is need for innovations to develop a low-cost method especially for developing countries which have a huge burden of these chronic viral infections. A multifaceted approach is required to improve the accuracy, reliability, and clinical utility of these tests.

Table I: Various FDA approved tests for viral quantification in clinical samples for HIV, HBV, HCV and CMV

Viruses	Assay	Principle	Detection range
HIV	Abbott RealTime HIV-1	Real time PCR	$40\text{-}1 \times 10^7$ copies/mL
	Roche Amplicor HIV-1 Monitor test	Real-time PCR	$400\text{-}7.5 \times 10^5$ copies/mL
	Cobas AmpliPrep/Cobas TaqMan HIV-1 test	Real time PCR	$48\text{-}1 \times 10^7$ copies/mL
	NucliSens HIV-1 QT	NASBA	$176\text{-}3.47 \times 10^6$ copies/mL
	Versant HIV-1 RNA 3.0	bDNA	$75\text{-}5 \times 10^5$ copies/mL
HBV	Cobas TaqMan HBV test	Real time PCR	$20\text{-}1.7 \times 10^8$ IU/mL
	Abbott real time HBV assay	Real-time PCR	$10\text{-}1 \times 10^9$ IU/mL
HCV	Cobas AmpliPrep/Cobas TaqMan HCV test	Real time PCR	$43\text{-}6.9 \times 10^7$ IU/mL
	Cobas Amplicor HCV test, v 2.0	Real time PCR	$600\text{-}5 \times 10^5$ IU/mL
	Versant HCV RNA 3.0 assay	bDNA	$3200\text{-}4 \times 10^7$ IU/mL
CMV	Cobas AmpliPrep/Cobas TaqMan CMV test	Real time PCR	$137\text{-}9.1 \times 10^6$ IU/mL
	Artus CMV RGQ MDx Kit	Real time PCR	$159\text{-}7.94 \times 10^7$ IU/ml

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Conflicts of interest

There are no conflicts of interest.

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