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Mini-Reviews

Quantitative Nucleic Acid Amplification Methods for Viral Infections

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BACKGROUND: Over the past 2 decades there have been substantial improvements in the methods used to quantify viral nucleic acid in body fluids and in our understanding of how to use viral load measurements in the diagnosis and management of patients with a number of viral infections. These methods are now integrated into a wide range of diagnostic and treatment guidelines and commonly deployed in a variety of clinical settings.

CONTENT: Quantitative nucleic acid amplification methods that are used to measure viral load are described along with key issues and important variables that affect their performance. Particular emphasis is placed on those methods used in clinical laboratories as US Food and Drug Administration–cleared or laboratory-developed tests. We discuss the clinical applications of these methods in patients with HIV-1, hepatitis C virus, hepatitis B virus, cytomegalovirus, Epstein-Barr virus, and BK polyomavirus infections. Finally, the current challenges and future directions of viral load testing are examined.

SUMMARY: Quantitative nucleic acid amplification tests provide important information that can be used to predict disease progression, distinguish symptomatic from asymptomatic infection, and assess the efficacy of antiviral therapy. Despite the advances in technology, large challenges remain for viral testing related to accuracy, precision, and standardization. Digital PCR, a direct method of quantification of nucleic acids that does not rely on rate-based measurements or calibration curves, may address many of the current challenges.

The development of quantitative nucleic acid amplification methods created new opportunities for clinical laboratories to impact the diagnosis and management of patients with viral diseases. Before the development of these methods, virologists were limited to either laborious culture-based methods available only in research laboratories or insensitive antigen assays to measure viral load. The term "viral load" first appeared in the literature in 1987 in a report by Jonas Salk proposing that viral load in HIV-1–infected individuals could be reduced by boosting the immune response, leading to reduced morbidity, mortality, and disease transmission *(1)*. Viral load assays assess the overall virus replicative activity that reflects the underlying disease process, usually by quantification of the viral nucleic acid in the blood. Although viral load testing in HIV-1 infection is an early example of how testing has increased our understanding of a disease process and improved patient care, viral load testing has had a similar impact on patients with many other viral infections.

Quantitative Nucleic Acid Amplification Methods

A variety of methods are used to quantify the amount of viral RNA or DNA in a clinical sample. The most commonly used methods in clinical laboratories include PCR, nucleic acid sequence–based amplification (NASBA),[2] and branched DNA (bDNA) assays.

Competitive PCR is a reliable and robust method that was the basis of the first generation of viral load assays for HIV-1 and hepatitis C virus (HCV) used in clinical laboratories. These assays, based on conventional standard PCR, are still in use by some clinical laboratories but are rapidly being replaced by real-time PCR methods. The basic concept behind competitive PCR is the coamplification in the same reaction tube of target and calibrator templates with equal or similar lengths and with the same primer binding sequences *(2)*. Because both templates are amplified with the same primer pair, identical thermodynamics and amplification efficiencies are ensured. The amount of the calibrator must be

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[2] Nonstandard abbreviations: NASBA, nucleic acid sequence-based amplification; bDNA, branched DNA; HCV, hepatitis C virus; Cq, fractional PCR cycle used for quantification in qPCR assays; qPCR, quantitative real-time PCR; T_m, melting temperature; TMA, transcription-mediated amplification; RT, reverse transcriptase; BKV, BK polyomavirus; CMV, cytomegalovirus; EBV, Epstein-Barr virus; HBV, hepatitis B virus; FDA, US Food and Drug Administration.

known and, after amplification, the products from both templates must be distinguishable from each other.

Real-time amplification and detection methods are particularly well suited for quantification of nucleic acid because the amount of the fluorescent signal generated is proportional to the concentration of the target DNA or RNA in the original sample. Real-time PCR and transcription-based amplification methods are the most commonly used quantitative methods. For real-time PCR, the fluorescent signal is measured during the exponential phase of amplification, which is where the amplification plot crosses the threshold. This is in contrast to standard PCR methods that measure the endpoint signal. There are advantages to measuring the fluorescent signal during the exponential phase of amplification; the reaction components are not limiting, and the assay is less sensitive to the effects of inhibitors. As a result, quantitative real-time PCR (qPCR) assays are more reproducible than standard end-point PCR assays. Both internal and external calibrators can be used with real-time assays, but the improved imprecision of real-time assays allows more reliable results to be obtained with an external calibration curve than would be obtained with standard PCR. When external calibrators are used, a calibration curve is generated by plotting the $\log_{10}$ concentration of the external calibrator vs the fractional PCR cycle used for quantification (Cq), and this plot is used to calculate the concentration of nucleic acid in the sample. The concentration of nucleic acid in the sample is inversely related to the Cq; the higher the concentration of the nucleic acid, the lower the Cq *(3)*. In general, quantitative real-time PCR (qPCR) assays are not more sensitive than standard PCR assays; however, they have a much broader linear range, typically 6–7 orders of magnitude.

In its simplest format, the real-time PCR product is detected as it is produced by using fluorescent dyes that preferentially bind to double-stranded DNA *(4)*. The dye will bind to both specific and nonspecific PCR products. The detection can be improved through melting curve analysis *(5)*. SYBR Green I was the first dye used in melting curve analysis. Immediately after the last PCR cycle, the amplicon is denatured, cooled to about 10 °C below the expected melting temperature (T_m), and heated at a slow ramp rate while the fluorescence is monitored continuously to look for a rapid decrease in fluorescence around the T_m of the amplicon. An individual amplicon has its own T_m, providing a simple closed-tube method for analysis.

The specificity of real-time PCR can also be increased by including fluorescent probes in the reaction mixture. These probes are labeled with fluorescent dyes or with combinations of fluorescent and quencher dyes. In hydrolysis probe PCR assays the 5′-to-3′ exonuclease activity of *Taq*DNA polymerase is used to cleave a nonextendable hybridization probe during the primer extension phase of PCR *(6)*. The use of dual hybridization probes is another approach to real-time PCR *(7)*. This method uses 2 specially designed sequence-specific oligonucleotide probes. These hybridization probes are designed to hybridize within 1–5 nucleotides apart on the product molecule. The 3′ end of the anchor probe is labeled with a donor dye, and the 5′ end of the reporter probe is labeled with an acceptor dye. The donor dye is excited by an external light source, and instead of emitting light, it transfers its energy to the acceptor dye by fluorescent resonance energy transfer. The excited acceptor dye emits light at a longer wavelength than the unbound donor dye, and the intensity of the acceptor dye light emission is proportional to the amount of PCR product.

Real-time detection and quantification of amplification products can also be accomplished with molecular beacons *(8)*. Molecular beacons are hairpin-shaped oligonucleotide probes with an internally quenched fluorophore whose fluorescence is restored when the probes bind to a target nucleic acid. The probes are designed in such a way that the loop portion of each probe molecule is complementary to the target sequence. The stem is formed by the annealing of complementary arm sequences on the ends of the probe. A fluorescent dye is attached to one end of one arm, and a quenching molecule is attached to the end of the other arm. The stem keeps the fluorophore and quencher in close proximity such that no light emission occurs. When the probe encounters a target molecule, it forms a hybrid that is longer and more stable than the stem and undergoes a conformational change that forces the stem apart, causing the fluorophore and the quencher to move away from each other, restoring the fluorescence.

A novel class of asymmetric, partially double-stranded, linear probes can be used in qPCR assays *(9)*. The partially double-stranded probe is composed of 2 complementary oligonucleotides of very different lengths. The long target-specific strand is 5′ labeled with a fluorophore and is blocked on the 3′ end to prevent extension. The shorter strand is complementary to the 5′ end of the long strand and a quencher dye attached to its 3′ end. In the absence of target, the quencher oligonucleotide hybridizes to the target-specific oligonucleotide and the duplex does not fluoresce, because of the close proximity of the reporter and quencher dyes. When the target is present, the long strand binds preferentially to the target, resulting in increased fluorescence due to separation of the reporter and quencher dyes. Partially double-stranded probes are better able to detect targets with a high level of genetic heterogeneity (e.g., HIV-1 and HCV) than are molecular beacons and hydrolysis probes, owing primarily to their increased length and less stringent hybridization conditions.

Digital PCR is the next advance in the evolution of quantitative PCR methods. PCR exponentially amplifies

nucleic acids, and the number of amplification cycles and the amount of amplicon allows the computation of the starting quantity of targeted nucleic acid. However, many factors complicate this calculation, often creating uncertainties and inaccuracies, particularly when the starting concentration is low. Digital PCR attempts to overcome these difficulties by transforming the exponential data from conventional PCR to digital signals that simply indicate whether or not amplification occurred *(10)*. An additional benefit of digital PCR is that it can provide absolute quantification of target nucleic acid without reference standard curves.

Digital PCR is accomplished by capturing or isolating each individual nucleic acid molecule present in a sample within many chambers, zones, or regions that are able to localize and concentrate the amplification product to detectable levels. After PCR amplification, a count of the areas containing PCR product is a direct measure of the absolute quantity of nucleic acid in the sample. The capture or isolation of individual nucleic acid molecules may be done in capillaries, microemulsions, or arrays of miniaturized chambers or on surfaces that bind nucleic acids. Digital PCR has many applications, including detection and quantification of low levels of pathogen sequences. It can provide a lower limit of detection than real-time PCR methods with better imprecision at very low concentrations. As opposed to relative quantification, digital PCR provides absolute quantification with no need for reference standards. Currently, digital PCR is used as a research tool but it may find applications in clinical laboratories to resolve ambiguous results obtained with qPCR assays or for creating accurate viral reference standards as the technology becomes less costly *(11, 12)*.

NASBA and transcription-mediated amplification (TMA), often referred to collectively as transcription-based amplification methods, are both isothermal RNA amplification methods modeled after retroviral replication *(13, 14)*. The methods are very similar in that the RNA target is reverse transcribed into cDNA and then RNA copies are synthesized with an RNA polymerase. NASBA uses avian myeloblastosis virus reverse transcriptase (RT), RNase H, and T7 bacteriophage RNA polymerase, whereas TMA uses an RT enzyme with endogenous RNase H activity and T7 RNA polymerase.

Amplification involves the synthesis of cDNA from the RNA target with a primer containing the T7 RNA polymerase promoter sequence. The RNase H then degrades the initial strand of target RNA in the RNA–cDNA hybrid. The second primer then binds to the cDNA and is extended by the DNA polymerase activity of the RT, resulting in the formation of double-stranded DNA containing the T7 RNA polymerase promoter. The RNA polymerase then generates multiple copies of single-stranded, antisense RNA. These RNA product molecules reenter the cycle, with subsequent formation of more double-stranded cDNA molecules that can serve as templates for more RNA synthesis. A 10^9-fold amplification of the target RNA can be achieved in <2 h by this method. NASBA has also been used with molecular beacons to create a homogeneous, kinetic amplification system similar to real-time PCR *(15)*. Real-time TMA-based quantitative assays are in development.

The bDNA signal amplification system is a solid-phase, sandwich hybridization assay incorporating multiple sets of synthetic oligonucleotide probes *(16)*. The key to this technology is the amplifier molecule, a bDNA molecule with 15 identical branches, each of which can bind to 3 labeled probes. Multiple target-specific probes are used to capture the target nucleic acid onto the surface of a microtiter well. A second set of target-specific probes also binds to the target. Preamplifier molecules bind to the second set of target probes and up to 8 bDNA amplifiers. Three alkaline phosphatase–labeled probes hybridize to each branch of the amplifier. Detection of bound labeled probes is achieved by incubating the complex with dioxetane, an enzyme-triggerable substrate, and measuring the light emission in a luminometer. The resulting signal is directly proportional to the quantity of the target in the sample. The quantity of the target in the sample is determined from an external standard curve.

Selected Clinical Applications of Viral Load Testing

Viral load testing may be clinically indicated in diagnosis, prognosis, and preemptive and therapeutic monitoring. These indications for patients with BK polyomavirus (BKV), cytomegalovirus (CMV), Epstein–Barr virus (EBV), hepatitis B virus (HBV), HCV, and HIV-1 infections are summarized in Table 1.

Viral load may predict disease progression. Probably the best example is HIV-1 viral load as a predictor of progression to AIDS and death in infected individuals. This predictive value was first demonstrated in 1996 as part of a multicenter AIDS cohort study *(17)*. The investigators showed that the risk of progression to AIDS and death was directly related to the magnitude of the viral load in plasma at study entry. The viral load in plasma was a better predictor of disease progression than the number of $CD4^+$ lymphocytes. Subsequent studies have confirmed that baseline viral load critically influences disease progression.

CMV viral load testing is useful for deciding when to initiate preemptive therapy in organ transplant recipients and for distinguishing active disease from asymptomatic infection. The level of CMV DNA can predict the development of active CMV disease, *(18)*, with higher viral load values increasing the risk of symptomatic disease. Currently, there are 2 qPCR assays cleared by the US

Table 1. Indications for viral load testing.[a]

Virus	Specimen type	Indication for testing	Appropriate time point or interval
BKV	Plasma or urine	Preemptive monitoring	Every 3 months up to 2 years posttransplant or with allograft dysfunction or biopsy evidence of BKV nephropathy
		Therapeutic monitoring	Baseline, then every 2–4 weeks
CMV	Whole blood or plasma	Preemptive monitoring	Solid organ and hematopoietic stem cell transplant patients, weekly during the highest risk period (12–14 weeks and 100 days posttransplant, respectively)
		Therapeutic monitoring	Baseline, then weekly while on therapy (viral load should become undetectable in plasma)
EBV	Whole blood, PBMC, or plasma	Preemptive monitoring	No consensus recommendations available for monitoring for PTLD[b]; strategies include frequent monitoring for first 3 months after solid organ or hematopoietic stem cell transplant with longer intervals thereafter
HBV	Plasma or serum	Diagnosis of HBeAg-negative CHB	In HBsAg-positive patients with ALT concentrations within the reference interval, every 3 months for 1 year, then every 6 months thereafter
		Decision to treat CHB	HBeAg-positive CHB; consider treatment if HBV DNA >20 000 IU/mL after 3–6 months and ALT persistently elevated
		Therapeutic monitoring	HBeAg-negative CHB; consider treatment if HBV DNA >2000 IU/mL and ALT persistently elevated;
			Baseline, week 12 and 24 of treatment, then every 3–6 months
HCV	Plasma or serum	Diagnosis of chronic infection	In HCV-seropositive individuals to demonstrate viremia; multiple samples may be required because viremia may be intermittent
		Therapeutic monitoring	Baseline, and subsequent times dependent on HCV genotype and expected kinetics of response to different therapies, particularly those that include direct acting antiviral agents.
HIV-1	Plasma	Disease progression	Magnitude of viral load predicts progression to AIDS and death in untreated individuals
		Diagnosis of acute infection	In HIV-seronegative individuals to document viremia
		Therapeutic monitoring	Baseline, 2–8 weeks after initiation of therapy and then every 36 months thereafter

[a] Modified with permission from table in the Appendix to *(30)*.
[b] PTLD, posttransplant lymphoproliferative disorder; HBsAg, HB surface antigen; HBeAg, HB "e" antigen; CHB, chronic HB.

Food and Drug Administration (FDA) for CMV (COBAS Ampliprep/COBAS TaqMan CMV Test, Roche Diagnostics, and artus RGQ Mdx, Qiagen) (Table 2). However, many clinical laboratories continue to use laboratory-developed tests. Present methods for measuring CMV viral load show considerable quantitative variability. Accuracy and imprecision are crucial to assay interpretation and establishing uniform thresholds for clinical disease attribution and for preemptive treatment strategies.

Similarly, EBV viral load testing plays an important role in monitoring and diagnosis of EBV-associated post-transplant lymphoproliferative disorder in organ transplant recipients *(19)*. There is no consensus on specimen type (whole blood, leukocytes, or plasma), gene target, or frequency of monitoring patients for EBV viral load, and no FDA-cleared test is currently available. Consequently, each center must establish their own interpretation and intervention guidelines.

BKV viral load monitoring is helpful in predicting the risk for developing BKV-associated nephropathy in kidney transplant recipients and assessing the efficacy of interventions to reduce kidney damage *(20)*. Although monitoring transplant recipients for BKV viremia is the

Table 2. FDA-approved viral load assays.

Virus	Assay (manufacturer)	Method	Gene target[a]	Dynamic range
HIV-1	Versant 3.0 (Siemens)	Branched DNA	*pol*	75–500 000 copies/mL
	COBAS Amplicor Monitor 1.5 (Roche)[b]	Competitive RT-PCR	*gag*	Standard: 400–750 000 copies/mL; ultrasensitive: 50–100 000 copies/mL
	NucliSens QT (bioMérieux)[b]	NASBA	*gag*	176–3 470 000 copies/mL
	COBAS Ampliprep/COBAS TaqMan 2.0 (Roche)	RT-qPCR	*gag, LTR*	20–10 000 000 copies/mL
	RealTime (Abbott)	RT-qPCR	*int*	40–10 000 000 copies/mL
HCV	Versant 3.0 (Siemens)	Branched DNA	*5′UTR*	615–7 700 000 IU/mL
	COBAS Amplicor Monitor 2.0 (Roche)[b]	Competitive RT-PCR	*5′UTR*	600–500 000 IU/mL
	COBAS Ampliprep/COBAS TaqMan Test 2.0 (Roche)	RT-qPCR	*5′UTR*	43–69 000 000 IU/mL
	RealTime (Abbott)	RT-qPCR	*5′UTR*	12–100 000 000 IU/mL
HBV	COBAS Ampliprep/COBAS TaqMan Test 2.0 (Roche)	qPCR	*precore/core*	20–170 000 000 IU/mL
	RealTime (Abbott)	qPCR	*surface*	10–1 000 000 000 IU/mL
CMV	COBAS Ampliprep/COBAS TaqMan Test (Roche)	qPCR	*UL54*	137–9 100 000 IU/mL
	artus RGQ Mdx (Qiagen)	qPCR	*MIE*	119–79 400 000 IU/mL

[a] *pol*, Polymerase, protease and integrase enzymes; *gag*, group-specific antigens or capsid proteins; *LTR*, long terminal repeats; *int*, integrase region of the polymerase gene; *5′UTR*, 5′ untranslated region; *precore/core*, precore/core proteins; *surface*, surface antigens; *UL54*, DNA polymerase; *MIE*, major immediate early gene.
[b] Not commercially available.

standard of care for most renal transplant programs, there is no FDA-cleared test, and a number of laboratory-developed tests are deployed in clinical laboratories with marked variability in BKV viral load measurements among commonly used tests *(21)*. This interassay variability complicates uniform application of BKV screening guidelines.

Viral load testing plays an increasing role in predicting and monitoring patient response to antiviral therapy. Quantitative tests for HIV-1 RNA are the standard of practice for guiding clinicians in initiating, monitoring, and changing antiretroviral therapy, and guidelines for their use in clinical practice have been published *(22)*. Although not FDA cleared for diagnosis, the HIV-1 viral load tests are featured in the recent HIV diagnostic testing algorithm proposed by the CDC *(23)*. In this algorithm, an HIV-1 viral load assay can be used to facilitate prompt diagnosis of acute HIV-1 infection and differentiate acute HIV-1 infection from false-positive immunoassay results when fourth generation screening and supplemental antibody test results are discordant.

There are 5 FDA-approved assays to quantify HIV-1 RNA in plasma, AMPLICOR HIV Monitor test (Roche Diagnostics), a conventional end-point RT-PCR assay; Versant HIV-1 RNA assay (Siemens Healthcare), a bDNA assay; NucliSens HIV EasyQ HIV-1 assay, an NASBA assay; COBAS Ampliprep/COBAS TaqMan HIV-1 test (Roche Diagnostics), an RT-qPCR assay using 5′ exonuclease hydrolysis probes; and RealTime HIV-1 test (Abbott Molecular), an RT-qPCR assay using partially double-stranded probes (Table 2). The newer RT-qPCR assays offer several advantages over the other methods. These assays are very sensitive (20–40 copies/mL) with a broad linear range (6 $\log_{10}$), have better inclusivity for all HIV-1 subtypes, and are less prone to carryover contamination.

The end point for HCV therapy is a sustained virological response, which is defined as undetectable HCV RNA in the blood 24 weeks after the end of therapy. Both viral load and genotype are independent predictors of response to combination therapy with pegylated interferon and ribavirin in chronic HCV infections, although genotype is the main predictor of response *(24)*. Those patients with high pretreatment viral load values (600 000 IU/mL) or genotype 1 infections have lower sustained response rates compared to those with genotype 2 and 3 infections. Monitoring of HCV RNA levels during treatment is key in determining the virologic response, in guiding the duration of treatment, and in deciding when to stop treatment. This is particularly important with development of the new direct-acting antiviral agents for treatment of hepatitis C *(25)*. Guidelines for monitoring patients on or that have completed therapy with regimens that include the direct-acting antiviral agents are evolving (http://www.hcvguidelines.org/). Also, HCV RNA testing has replaced the recom-

binant immunoblot assay as a supplemental test for the diagnosis of HCV infection *(26)*.

There are 4 FDA-cleared HCV viral load assays, the AMPLICOR HCV Monitor test (Roche Diagnostics), a conventional end-point RT-PCR assay; Versant HCV assay (Siemens Healthcare), a bDNA assay; COBAS Ampliprep/COBAS TaqMan HCV test (Roche Diagnostics), a RT-qPCR assay using 5′ exonuclease hydrolysis probes; and RealTime HCV test (Abbott Molecular), an RT-qPCR assay using partially double-stranded probes (Table 2). The newer RT-qPCR assays are widely deployed in clinical laboratories, offer very low limits of quantification (12–43 IU/mL), have broad dynamic ranges ($\geq$6 $\log_{10}$), and lack significant genotype bias.

Viral load assays have also been used in monitoring response to therapy in patients chronically infected with HBV *(27)*. There are 2 FDA-cleared qPCR viral load assays for HBV available from Abbott and Roche (Table 2). In organ transplant recipients, the persistence of CMV viral load after several weeks of antiviral therapy is associated with the development of resistance *(28)*.

Current Challenges

Over the years there has been a transition from the use of laboratory-developed tests to FDA-cleared tests to measure viral load as these tests became increasing available. In addition, WHO international standards and reference panels are now available for HIV-1, HCV, HBV, CMV, and EBV. Together, these advances have helped improve reproducibility, both within and between laboratories, but considerable variation still exists between the results for some assays *(29)*.

Sources of assay variability in viral load assays include reagents used for detection of amplified product, target gene selection (genotype bias), nucleic acid extraction method, and use of laboratory-developed tests *(29)*. Because of this variation, published case series from individual centers demonstrating the clinical utility of quantitative nucleic acid amplification tests for many viruses are not widely applicable. With the important exceptions of HIV-1 and HCV infections, viral load testing thresholds for risk stratification and therapeutic decisions are largely absent. The reduction of the inherent variability in viral load measurements will require a multifaceted approach to improve the accuracy, reliability, and clinical utility of these tests. The CLSI has published guidelines for quantitative molecular methods for infectious diseases that address the development, verification, validation, and application of quantitative PCR assays and other nucleic acid amplification methods for infectious diseases *(30)*.

Digital PCR may provide an opportunity to reduce the quantitative variability associated with real-time PCR methods because it does not rely on rate-based measurements (Cq values) or calibration curves. However, methods may need to be further optimized to match the analytical sensitivity of real-time PCR methods.

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