HIV-1 Load Quantitation: A 17-Year Perspective

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During the past decade and a half, quantitation of plasma-associated human immunodeficiency virus type 1 (HIV-1) RNA level, or HIV-1 load, has been validated in clinical practice and clinical trials as an important surrogate marker of HIV-1 disease progression and of the potency and durability of antiretroviral regimens. This review highlights some of the history, accomplishments, and impact of Tom Merigan’s laboratory on the use of HIV-1 load as a marker, as well as on updating technologies for determining HIV-1 load, their performance, interpretation of the results, and their use in clinical practice.

Although my introduction to Tom Merigan had been a year earlier, during my Infectious Diseases fellowship interviews at Stanford University, nothing prepared me for the laboratory experience I was about to start in 1988. It was a time during the infancy of HIV-1 clinical trials, when azidothymidine (zidovudine) monotherapy had just been found to decrease HIV-associated morbidity and mortality [1] and was then currently being evaluated in the AIDS Clinical Trials Group protocols 016 and 019. Other than clinical end points such as newly diagnosed AIDS-defining illnesses or death, CD4+ T cell counts, p24 antigen levels, and, later, quantitative HIV-1 culture results were the only measures we had of the effectiveness of what we were later to learn was a minimally potent antiretroviral agent when used as monotherapy. We were desperate for better markers of antiviral response. A decade earlier, Tom and his group had discovered the usefulness of virus markers as a measure of the effect of interferon therapy for hepatitis B virus (HBV) [2]. Quantitation of circulating HBV particle–associated DNA polymerase activity, hepatitis B core antigen, and HBV particle–associated DNA and the subsequent demonstration of declines in these HBV markers with interferon treatment had given him insight into what might work in monitoring HIV-1 treatment.

I was introduced to the concept of the polymerase chain reaction (PCR) by Tom early after its discovery and reported use in 1988 for the detection of HIV-1 proviral DNA in peripheral blood mononuclear cells (PBMCs) [3]. Few studies up to that point had used PCR technology or investigated its application as a diagnostic or therapeutic tool. However, 1988 was a watershed year, as several groups began publishing results about the use of PCR to measure HIV-1 DNA as a diagnostic test for HIV-1 infection, particularly in neonatal and pediatric cases [4, 5]. Because most of this early work involved PBMCs, a natural extension was the description of HIV-1 transcriptional activity in PBMCs, using reverse transcriptase (RT)–PCR to detect HIV-specific mRNA [6]. The detection and quantitation of HIV-1 particle–associated RNA in blood serum or plasma remained elusive.

I had been working diligently in Tom’s laboratory on reproducing the previously published PCR assays for HIV-1 DNA on PBMCs. Although these assays were qualitative by design, it seemed plausible that a PCR assay could be made quantitative and that we could then potentially measure a change in proviral DNA level in PBMCs after initiation of antiretroviral treatment. The development of such an assay proved to be difficult at that point. After several discussions with Tom, he...
felt that I should spend some time at the Cetus Corporation in Emeryville, California, which, at the time, was the engine behind the burgeoning use of PCR technology. Through Tom’s ongoing collaboration and contact with Cetus scientists, I was fortunate to be able to spend the next year at Cetus working with some great molecular biologists and other scientists.

It was known at the time that HIV-1 could be cultured from blood plasma and, therefore, exist in a cell-free state. It was further known that the infectious titer in plasma correlated with stage of disease [7, 8]. However, we and others began to realize that quantitative culture methodology for both cell-associated and cell-free HIV-1 required 2–3 weeks to obtain results, had high labor and reagent costs, required fresh donor PBMCs for propagation of virus, and had limited sensitivity and poor reproducibility. For those reasons, we decided to concentrate on developing an assay that would be less labor intensive and less costly. We focused on quantitation of HIV-1 RNA in serum or plasma, using PCR techniques, in the hope that RNA levels would also correlate with the stage of HIV-1 disease and response to treatment. PCR-based assays at the time required radioactive isotopes incorporated into primers or probes and gel electrophoresis to detect amplicons. We understood early on that broader application of any plasma RNA assay required a nonisotopic detection and quantitation scheme and, preferably, a microplate format to achieve the higher throughput necessary to evaluate the multiple samples from clinical trials and eliminate the need for electrophoretic equipment.

Although my early development work took place at Cetus, by early fall of 1989, I had established a working nonisotopic HIV-1 load assay at Stanford.

My daily commute to Emeryville was cut short on 17 October 1989, when the Loma Prieta earthquake struck the San Francisco Bay area. Commuting was no longer an option, because the Bay Bridge was out of commission, and it was taking me several hours a day just to go back and forth. Because Tom’s laboratory had all the necessary equipment by then, I was able to establish all of the necessary conditions for continued development of the HIV-1 RNA assay solely at Stanford. Because additional antiretrovirals were coming on the horizon, Tom was anxious to make progress on a quantitative viral RNA assay.

Our preliminary data correlating stage of HIV-1 disease and HIV-1 load was beginning to look promising. Along with our Cetus collaborators, we presented these first findings at the UCLA Keystone Meeting in 1990 and, subsequently, at the International AIDS Conference in San Francisco that same year. This work culminated in our first publication on the correlation between HIV-1 load and stage of HIV-1 disease in 1991 [9]. Using patient serum samples from Stanford-affiliated clinics, we knew in early 1990 that we could show reductions in plasma HIV-1 loads in some, but not all, patients who had received zidovudine monotherapy. We needed to test blood samples from more patients to validate these findings. I tested plasma samples from patients enrolled in clinical trials at the time. However, I was unable to get any detectable PCR product from any of them. I repeated my experiments and still did not get an amplicon. I went back to some of the serum samples I had previously tested, along with EDTA plasma samples, and the assay worked beautifully. Something appeared to be inhibiting my PCR experiments when I used heparin plasma samples and not serum. Most of the clinical trial blood samples at the time were collected in blood collection tubes containing heparin as an anticoagulant, because it was thought that HIV-1 culture and flow cytometry techniques for enumeration of T cell subsets were best performed in heparinized blood. The investigation into why samples containing heparin resulted in RT-PCR inhibition created a 6-month diversion for us. It was certainly known that heparin could be used to isolate RT and DNA polymerase on heparin-coated sepharose columns and could directly inhibit DNA polymerase function, which was essential for PCR to work [10–12]. Using reconstruction experiments in which HIV-1–infected U1 cells were spiked into HIV-1–seronegative blood samples and HIV-1–infected patient samples collected in blood collection tubes containing different anticoagulants, we, along with our Cetus collaborators, found that the HIV-1 load assay worked well in all of them except heparin-containing samples. Furthermore, we found that the inhibitory effect of heparin on the PCR was also concentration dependent. Finally, we could demonstrate reversal of the heparin–associated inhibition of PCR by the addition of heparinase [13].

Armed with this new knowledge, we set about analyzing new plasma samples collected in EDTA- or citrate-containing blood collection tubes from patients at Stanford-affiliated clinics who had initiated antiretroviral therapy. Although already in 1990 we had internally made the observation that zidovudine therapy resulted in modest reductions in HIV-1 load, another group published these very findings in September of 1991 with 4 patients [14]. Our findings were published in November of 1991, in which we showed that, among 72 patients, those currently receiving antiretroviral treatment had significantly lower HIV-1 loads than did untreated patients and that combination therapy with zidovudine and didanosine resulted in significantly greater reductions in HIV-1 load than did zidovudine monotherapy [15]. Our observation was the first significant demonstration that quantitation of plasma HIV-1 load could be used to show reductions with antiretroviral treatment and to define differences in the potencies of different antiretroviral regimens. More than a decade later, it looked like Tom had again found the virus marker he was looking for.

As if the heparin story was not enough of a distraction for us, I decided, against Tom’s wishes, to use our newly created assay to detect and quantitate HIV-1 load in other body compartments besides blood. With the help of an energetic Stanford
medical student, Jonathan Mermin, we were able to detect and correlate HIV-1 loads in seminal fluid and blood plasma with CD4+ T cell count [16]. Although Tom was not initially interested in our pursuit of genital tract pathogenesis–based questions, this project served as the foundation for several more papers from the Stanford group demonstrating antiretroviral response and the presence of antiretroviral resistance mutations in HIV-1 strains obtained from genital secretions [17, 18].

I left Tom’s laboratory in 1991 to pursue my own career in infectious diseases with Stanford University and the Department of Veterans Affairs. However, we were granted two US patents for our development of HIV-1 load assays to monitor antiretroviral treatment. In addition, our original description in 1991 of the correlation between HIV-1 load and stage of disease was recently recognized by the editors of this journal as being one of the most significant papers published in the Journal of Infectious Diseases in the past 100 years [19]. For the past 15 years, I have continued to maintain research interests in the evaluation of new diagnostic and therapeutic monitoring assays, which Tom had introduced me to in 1988. Herein, I briefly review some additional historical events and recent important findings in HIV-1 load testing.

**METHODOLOGY FOR DETERMINATION OF HIV-1 LOAD**

**Available assays.** Several methods of quantifying HIV-1 load have now been developed and are reviewed elsewhere [20]. Briefly, 3 methods are currently approved by the US Food and Drug Administration (FDA) for the assessment of risk of HIV-1 disease progression or response to antiretroviral treatment. They include the HIV RNA RT-PCR assay (Amplicor HIV-1 Monitor, version 1.5; Roche Molecular Systems), the branched-chain DNA assay (bDNA; Versant HIV RNA 3.0 Assay; Bayer Diagnostics), and nucleic acid sequence–based amplification (NASBA; NucliSens HIV QT; bioMérieux). An updated version of the NASBA assay, called NucliSens EasyQ (bioMérieux), uses real-time NASBA amplification and molecular beacon detection technology with a range of 50–3 million copies/mL. The NucliSens EasyQ assay is currently not approved by the US FDA for monitoring HIV-1 load. However, comparison of the EasyQ assay using the miniMag manual nucleic acid extraction method with the Roche COBAS Ampliprep/Standard HIV-1 Monitor yielded comparable results [21]. Another comparison of automated nucleic acid extraction, using Ampliprep/Ultra-sensitive HIV-1 Monitor and the Versant bDNA, version 3.0, also yielded comparable results [22]. Thus, all 3 conventional technologies produce similar HIV-1 load results. The addition of automated nucleic acid extraction will improve throughput and eliminate operator fatigue and the potential for error at the critical first step of the assay. The LCx assay (Abbott Laboratories) also quantifies HIV-1 RNA in plasma, using competitive RT-PCR followed by a microparticle EIA, and includes an internal control [23, 24]. In addition, Abbott and Celera Diagnostics have developed a real-time PCR assay for HIV-1 quantification [25]. Both assays are available in Europe and are not currently available in the United States. Additional methods for HIV-1 RNA detection include transcription-mediated amplification (Procleix HIV-1/HCV nucleic acid test; Gen-Probe), COBAS Ampliscreen HIV-1 (Roche Molecular Systems), and the UltraQual HIV-1 RT-PCR assay (National Genetics Institute), which are all approved by the US FDA to screen blood products for HIV-1.

A recently described approach for quantifying HIV-1 load is to measure virion-associated HIV-1 RT activity. The ExaVir Load (Cavidi Tech) is a quantitative HIV-1 RT assay that measures the amount of DNA made by HIV RT from virions contained in plasma, by a process similar to standard EIAs. The amount of DNA produced, in femtograms per milliliter, is then converted to number of virus copies per milliliter. This assay has a linear range of 400–1,250,000 copies/mL, and, although a longer assay time is required, it is somewhat less costly than traditional HIV-1 load tests. In recent studies, RT activity was found to be highly correlated with number of virus copies from currently available assays [26–28]. Recently, a more sensitive version of the assay has been developed that better quantitates samples with HIV-1 loads between 50 and 400 copies/mL [29].

**Sample handling and preparation.** For each of the commercially available assays, plasma should generally be collected in tubes containing the anticoagulant EDTA, either in standard blood collection tubes or plasma preparation tubes. Acid citrate dextrose or, in the case of the NucliSens assay, heparin-containing tubes can also be used. Although previous data from our group indicated a general equivalence in HIV-1 load results from samples collected in different tube types [30], recent data suggest that HIV-1 load results derived from different tube types may not be equivalent. In a recent pivotal clinical trial, critical differences in the numbers of subjects who achieved and sustained undetectable HIV-1 loads as determined by the HIV-1 Monitor assay were seen when plasma was collected in standard EDTA versus plasma preparation tubes [31].

Technical differences in centrifugation time and speed, particularly as suggested by the manufacturer of the Amplicor HIV-1 Monitor assay, and handling instructions for plasma preparation tubes, as well as specific handling procedures of plasma acquisition, probably contributed to these differences. Recent studies suggest that centrifugation of plasma preparation tubes followed by freezing of separated plasma within the tubes, rather than immediate separation and transfer to a new tube and subsequent freezing, resulted in a greater number of samples having discordant and detectable HIV-1 loads in patients with previously undetectable (<50 copies/mL) HIV-1 loads [32, 33]. This observation has prompted Becton Dickinson, the
manufacturer of plasma preparation tubes, to suggest that plasma should be removed from the tubes and transferred to a fresh, clean tube prior to freezing. Another study suggests that there does not appear to be any significant difference in HIV-1 loads determined using the VERSANT bDNA assay when blood is collected in plasma preparation tubes or standard EDTA collection tubes [34].

We had previously demonstrated that platelet-associated HIV-1 contributes a small but significant amount toward HIV-1 load quantitation. We also found that, when plasma preparation tubes were centrifuged at higher centrifugal force than current recommendations, HIV-1 load results obtained using the VERSANT bDNA assay were very similar to those derived from standard acid citrate dextrose blood collection tubes [35]. Recent data in our laboratory also indicate that doubling centrifugation time from 10 to 20 min and increasing centrifugal force from 1100 to 1500 g results in lower HIV-1 loads or in a greater number of samples collected in plasma preparation tubes that have undetectable HIV-1 loads or HIV-1 loads that are comparable to those in samples collected in standard EDTA tubes when the HIV-1 Monitor RT-PCR assay is used, compared with when plasma preparation tubes are processed in accordance with the package insert (author’s unpublished data).

Another group has also recently demonstrated that erythrocyte-associated HIV-1, in the presence of undetectable plasma HIV-1 load, can be a significant source for determination of HIV-1 load, particularly when samples may not have been centrifuged and separated appropriately [36]. Thus, platelet- and red blood cell–associated HIV-1 particles, which may not be efficiently removed by gel barrier separation at lower centrifugal forces, or without careful handling of standard blood collection tubes, may contribute to discordant HIV-1 load results between blood collection tubes [37]. Although, outside of clinical trials, the significance of these findings for clinical practice is unclear, sample processing factors may contribute to intermittently detectable HIV-1 loads (i.e., “blips”), which have been reported to have resulted in changes in antiretroviral regimens for some patients [33].

Although manufacturers of current assays recommend that plasma should be separated and frozen at −20°C to −70°C within 4–6 h of collection, to ensure maximum stability of RNA, studies from our group and others have demonstrated that, once blood has been centrifuged and plasma has been separated, these plasma samples can be held at room temperature for a number of hours without significant loss of HIV-1 RNA [30, 34, 38].

Recent studies indicate that valid and reproducible HIV-1 load results can be achieved using dried whole blood or plasma spots on filter paper [39]. This would eliminate the need for immediate freezer storage and expensive shipment to referral laboratories and would advance the use of these assays in resource-poor areas. Lloyd and colleagues have shown that, with Sample Tanker (Research Think Tank), larger volumes of plasma (up to 1 mL) can be dried on a cellulose acetate matrix and yield HIV-1 load results comparable to those obtained with frozen samples [40]. In addition, we have shown that multiple parameters (including HIV-1 and hepatitis C virus load, hepatitis C virus genotype, and HIV-1 genotypic and phenotypic resistance assays) can be assessed from a single dried sample. This matrix offers the advantage of using standard mail or commercial shipment without the use of dry or wet ice and the requirement for special biocontainment vessels and hazardous shipment designation. The average cost for commercial shipment of biohazardous material ranges from $50–$100 for US domestic shipping to several hundred dollars for international shipping. Transition to dried blood shipment for virological testing would result in millions of dollars saved in shipping costs. As more countries respond to the global AIDS crisis and the World Health Organization “3 by 5 Initiative” to initiate antiretroviral therapy, the need for virological assays will increase. Limited resources should not be used to pay for specialized sample shipment. Collection systems such as Sample Tanker or filter paper technologies will help clinical trials and clinical practice to proceed in these areas by allowing samples to be dried, temporarily stored, and sent to regional or international reference laboratories with the assurance that results are valid and reproducible.

**HIV-1 LOAD AND TREATMENT CONSIDERATIONS**

The Department of Health and Human Services guidelines for antiretroviral treatment of adults has incorporated HIV-1 load thresholds for when antiretroviral treatment should be initiated. Current guidelines suggest that initiation of antiretroviral treatment should be considered when the CD4+ T cell count is <350 cells/µL regardless of HIV-1 load, whereas, if CD4+ T cell counts are ≥350 cells/µL, antiretroviral treatment should be started only if the HIV-1 load is >100,000 copies/mL [41]. Although US FDA approval of HIV-1 load assays does not include an indication for using HIV-1 load testing in the diagnosis of HIV-1 infection, current Department of Health and Human Services guidelines suggest that HIV-1 load testing can be used for those patients with symptoms consistent with acute HIV infection and in whom HIV antibody tests yield negative or inconclusive results. Recent data suggest that early HIV-1 load testing can improve the diagnosis of HIV-1 infection during acute infection in a small number of patients [42].

In general, those patients who have not achieved a reduction in HIV-1 load of 0.5–0.75 log₁₀ copies/mL by 4 weeks, a reduction of 1 log₁₀ copies/mL by 8 weeks, or an undetectable HIV-1 load (<50 copies/mL) by 15–24 weeks should be considered for a modification in treatment. Patients who achieve
an undetectable HIV-1 load (<50 copies/mL) but experience a rebound in HIV-1 load to detectable levels should first have the HIV-1 load results confirmed. Recent data indicate that low-level intermittent viremia, defined as HIV-1 loads of 50–500 copies/mL, occurs frequently, is more likely related to assay and statistical variability, is unlikely to result in virological failure, and is not related to poor adherence issues [43, 44]. If the HIV-1 load rebound is sustained and within 50–5000 copies/mL, current guidelines suggest that short-term observation, rather than an immediate change in treatment, could be recommended. Recent data suggest that patients with sustained low-level viremia do not experience disease progression and may perhaps benefit immunologically from persistent low-level replication [45, 46]. If rebound in HIV-1 load is greater or, after initial observation, increases to >5000 copies/mL, a change in antiretroviral regimen is indicated. However, recent observations from the Collaborations in HIV Outcomes Research/United States (CHORUS) cohort indicate that patients with detectable HIV-1 loads of <20,000 copies/mL are at no greater risk for HIV-1 disease progression, even after antiretroviral treatment is adjusted for [47].

Older data in the absence of antiretroviral therapy suggested that HIV-1 load was important in risk assessment for progression of HIV-1 disease. More-recent studies have also determined that HIV-1 load after initiation of antiretroviral treatment continues to be an important marker for predicting disease progression [48, 49]. The HIV-1 load after 4 weeks of antiretroviral treatment has recently been found to be predictive of an undetectable HIV-1 load after 24 weeks of treatment. Patients with an HIV-1 load of >1000 copies/mL 4 weeks after starting treatment are unlikely to have a sustained virological response [50]. Emerging data indicate that there is no significant difference in HIV-1 load response in patients with non–subtype B HIV-1 who initiate antiretroviral treatment [51]. However, older patients (>50 years) are more likely to achieve an undetectable HIV-1 load, despite a less vigorous immune response and higher risk for clinical progression [52]. Finally, many publications have described the resultant HIV-1 load rebound after interruption of antiretroviral treatment. Recent data indicate that this rebound happens within days of treatment interruption, and in children it is often associated with significantly higher HIV-1 loads, compared with pretreatment HIV-1 loads [53, 54].

OTHER FACTORS AFFECTING HIV-1 LOAD

Providers should be aware of many clinical or patient-specific factors that may or may not affect quantification of HIV-1 load. In general, patients infected with antiretroviral-resistant virus tend to have lower HIV-1 loads than do those infected with wild-type virus [55], and women and African Americans have lower baseline HIV-1 loads when stratified by CD4+ T cell count than do white men, despite similar rates of clinical progression [56, 57]. Approximately 7% of patients achieve an undetectable HIV-1 load (defined as <400 copies/mL) after seroconversion; this is more likely to occur in patients with higher CD4+ T cell counts, women, and those who have a lower first HIV-1 load after diagnosis during acute infection [58]. HIV-1 load is not correlated with neuropsychological functioning [59] but, in the absence of highly active antiretroviral therapy, is strongly correlated with patient weight and degree of weight loss [60]. Furthermore, despite having differing effects on HIV-1 disease progression, GB virus and hepatitis B and C virus infections do not affect HIV-1 loads or responses to highly active antiretroviral therapy, compared with those in patients without any of these viral coinfections [61–63]. However, infections such as tuberculosis are associated with increased HIV-1 loads compared with those in uninfected control subjects and, despite antituberculosis treatment, can remain elevated over pretreatment levels [64]. Although prior studies demonstrated significant transient increases in HIV-1 load after routine immunization, newer studies indicate that, in patients receiving antiretroviral treatment, there is generally no significant increase in HIV-1 load after immunization [65]. Finally, although lower HIV-1 loads have been associated with a lower risk of mother-to-child and heterosexual transmission of HIV-1 [66, 67], low or undetectable HIV-1 loads have also been associated with the perception of decreased risk and increased frequency of unprotected anal intercourse among men who have sex with men [68, 69], emphasizing the need for better prevention education strategies.

SUMMARY

Quantification of HIV-1 load continues to be an important diagnostic and therapeutic test for use in clinical practice, clinical trials, and blood screening. The contribution of Tom Merigan to our understanding of using HIV-1 load data cannot be overemphasized. New technologies will continue to improve accuracy, detection of more diverse viral subtypes, turnaround time, and, it is hoped, costs, so that areas with limited resources can use this tool as HIV-1 treatment becomes more available. Health care providers should remain aware of emerging trends (e.g., changes in assays, including supersensitive assays with quantitation below 50 copies/mL; discordant responses; comorbid conditions, including recreational drug use; and use in occupational or postsexual exposure) that could affect HIV-1 load quantitation and, therefore, clinical decision making.

References


