

# CAP TODAY

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## Molecular assays in HIV-1 Dx and therapeutic monitoring

CAPTODAY and the Association for Molecular Pathology have teamed up to bring molecular case reports to CAPTODAY readers. Here, this month, is the fourth such case. (See the February, August, and September 2013 issues for the first three.) AMP members write the reports using clinical cases from their own practices that show molecular testing's important role in diagnosis, prognosis, treatment, and more. Case report No. 4 comes from the Department of Pathology and Laboratory Medicine, Hospital of the University of Pennsylvania. If you would like to submit a case report, please send email to the AMP at [amp@amp.org](mailto:amp@amp.org). For more information about the AMP, visit [www.amp.org](http://www.amp.org).

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HIV-1 viral load determinations are important to help direct proper therapy, and in special cases, to detect or confirm HIV-1 infection. We report a case of a patient with repeatedly positive HIV-1 serologic testing, yet consistently undetectable levels of HIV-1 virus, even though the patient was not receiving antiretroviral therapy. These results created clinical confusion for several years until resolved by the use of a more sensitive HIV-1 RNA assay. The case highlights the importance of molecular assays in HIV-1 diagnosis and prognostication, as well as several caveats of molecular testing in HIV-1.

### Introduction

The Centers for Disease Control and Prevention estimates that approximately 1.1 million people in the United States are living with HIV-1.<sup>1</sup> Traditional screening for HIV-1 has been performed by enzyme immunoassay, with repeatedly positive samples retested using a more specific test, usually Western blot, which can detect IgG class antibodies to HIV-1 proteins, as soon as six weeks after initial infection. (See references 2 and 3 for an in-depth explanation of these assays and newer screening algorithms.) After the initial infection, HIV-1 RNA levels rapidly increase to a peak level (as high as  $10^7$  copies/mL) that corresponds with seroconversion; therefore, in a case of a new infection with positive serological testing, one would expect a high



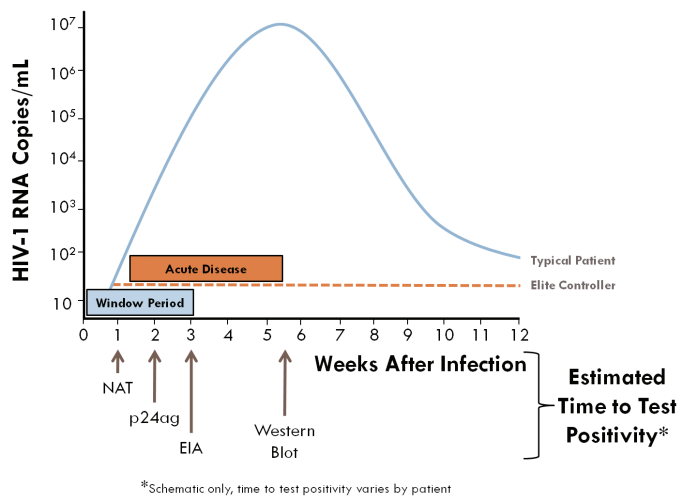
viral load (Fig. 1). After acute infection, patients develop either 1) a steady state of a high HIV-1 RNA viral load, which is associated with rapid disease progression, or 2) a U-shaped curve of HIV-1 RNA viral

load, with initial suppression of HIV-1 RNA viral load for a variable length of time associated with clinical latency, followed by an increasing viral load that corresponds with decreasing CD4 cell count and clinical decline.<sup>4</sup>

### Patient case

The clinical laboratory was consulted about a 47-year-old male who was being evaluated for a liver transplant. As part of the workup, serological evidence of HIV-1 infection was discovered; HIV-1 antibodies were detected by an enzyme immunoassay and confirmed by HIV-1 Western blot testing. Although the patient did not have clinical signs and symptoms of AIDS, or a definitive history of HIV exposure, additional testing was pursued. The patient's CD4 count was within normal limits. An HIV-1 viral load test was performed and reported  $<75$  copies/mL of HIV-1 RNA, the assay's lower limit of detection. Given these findings, the patient was followed clinically without initiation of antiretroviral therapy (ART).

Over the following two years, the patient underwent additional HIV-1 serological testing, all of which confirmed the presence of HIV-1 antibodies. However, several qualitative and quantitative molecular assays remained negative for HIV-1 RNA and DNA. Given the apparent inconsistency in the clinical data, additional viral load



**Fig. 1 Schematic of HIV-1 viral load versus HIV-1 assay positivity**

For most patients, HIV-1 viral load rises after infection, with a peak of as high as  $10^7$  copies/mL at approximately six weeks post-infection (blue line), which often corresponds to seroconversion. About 50 to 70 percent of HIV-1 infected people are symptomatic during this period (orange box denoting acute disease). During the first three to four weeks of infection, the patient has a rising viral load titer but no identifiable antibodies by enzyme immunoassay testing. This is the serologic window period. As demonstrated by the arrows at the bottom of the graph, testing by various assays becomes initially positive at various times during infection. Molecular assays for HIV-1 DNA or RNA (NAT, nucleic acid testing) may become positive as soon as one week after initial infection, allowing testing during the serological window period. p24 antigen testing may be positive in approximately two to three weeks, while third- and fourth-generation antigen/ELISA antibody testing can become positive in three to four weeks. Western blot testing is not positive until five or six weeks after infection. A rare subset of patients (less than one percent), called elite controllers, will develop antibodies to HIV-1 but have extremely low-level or undetectable viral loads ( $<50$  copies/mL, dashed orange line representing typical HIV-1 RNA viral load in these patients). These elite controllers are asymptomatic and have the same viral control as patients receiving antiretroviral therapy.

testing was performed using a highly sensitive RT-PCR platform for HIV-1 RNA, with a limit of detection of 20 copies/mL. This test revealed a viral load of 37 copies/mL. Based on the patient's positive serological testing, extremely low viral load, and normal CD4 count, he was diagnosed as an elite HIV-1 controller, without the need for ART. He is currently listed for liver transplantation.

### Discussion

Molecular testing has several applications in the management of HIV-1 infection, including therapeutic monitoring, diagnosing acute HIV-1 infection, and identifying elite controllers.

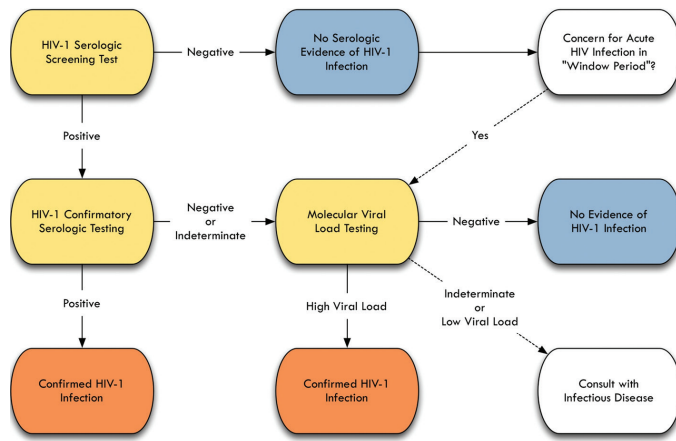
According to current U.S. (but not World Health Organization) treatment recommendations, ART should be offered to all adults with a typical HIV infection, regardless of CD4 count.<sup>5</sup> After initiation of ART, HIV-1 RNA levels should be monitored at least once every three months until the viral load is undetectable by standard assays (that is,  $<50$  copies/mL), which should occur by 24 weeks with effective therapy. From then on, HIV-1 RNA levels should

be monitored at least every three months to confirm continued suppression of viremia. Patients with sustained elevation of HIV-1 RNA ( $>50$  copies/mL) require evaluation for mechanisms of treatment failure, such as developed drug resistance.<sup>5</sup>

Although great improvements have been made over the past decade in detecting early HIV infection, including the introduction of third-generation antibody assays that detect both IgG and IgM HIV-1 antibodies and fourth-generation antigen/antibody combination assays, there remains a window period in which patients are infected with HIV-1 but not positive by serological assays (up to three to four weeks after infection) or Western blot (up to five to six weeks) (Fig. 1). So in addition to viral load monitoring, molecular assays have an important role in diagnosing acute HIV-1 infection. For adults, many diagnostic algorithms for HIV include HIV-1 RNA molecular testing as an adjunct when serological testing is unclear, or when there is high clinical suspicion for acute HIV-1 infection during the window period (Fig. 2). Viremia may be detected as soon as a week after infection, so HIV-1 RNA viral load testing can be useful, though not always essential, in detecting very early acute HIV-1 infection (Fig. 1), when some or all serologic tests are negative.

Although similar testing algorithms are useful for children age 18 months and older, testing in infants represents a different clinical situation because **1**) maternal HIV-1 antibodies may persist for several months and **2**) maternal antiretroviral therapy may lead to an undetectable viral load (HIV-1 RNA) in an infected infant. Currently, because of these factors, HIV-1 nucleic acid testing is the recommended approach for babies born to seropositive mothers, with the caveat that although viral RNA-based assays can have equal (or greater) sensitivity when compared with the DNA-based assays (i.e. detection of integrated HIV-1 proviral DNA in infected cells), HIV-1 RNA PCR assays can be affected by maternal ART or infant antiretroviral prophylaxis.<sup>6</sup>

In adult testing, high viral loads are expected at seroconversion, and lower but detectable viral loads in chronic infection; however, viral loads may very rarely not be detected at all and can lead to clinical confusion. Some patients are repeatedly positive by HIV-1 serological testing but have non-quantifiable or extremely low-level ( $<50$  copies/mL) viremia, have normal CD4 cell counts, and are clinically asymptomatic, without receiving therapy. These patients are considered elite controllers/elite suppressors and have HIV-1 control that is similar to that of those taking ART. This rare phenotype is seen in less than one percent of untreated HIV-1 patients, with unclear etiology. Elite controllers are different from long-term nonprogressors, a larger subset of patients who are able to maintain normal CD4 counts for long periods, but who still have detectable, albeit lower level, viremia, compared with



**Fig. 2 Generalized diagnostic testing algorithm for HIV-1**

A serological screening test (usually a third- or fourth-generation enzyme immunoassay) is performed first. Positive screening tests are confirmed by Western blot or other confirmatory serologic assay. In cases of unclear serological testing, where the confirmatory test is either negative or indeterminate, or in cases of high clinical suspicion of acute HIV-1 when the screening test is negative, molecular testing for HIV-1 RNA viral load can be performed. If no HIV-1 RNA is detected, infection is unlikely (although if there is high clinical suspicion of acute disease, repeat testing may be appropriate). If HIV-1 RNA is detected at a high level, HIV-1 can be diagnosed. Indeterminate HIV-1 viral load results or inconsistent results (i.e. a low or undetectable viral load with repeat positive serologies) may require consultation with an HIV specialist and the clinical laboratory.

normal patients. Elite controller status appears to be determined by the genetic factors in the patient, leading to robust cell-mediated immunity.<sup>7</sup>

In the presented case, it may appear that the final viral load result of 37 copies/mL is inconsistent with the previously reported “undetected” HIV-1 RNA by other assays. Examination of the analytical sensitivities of the previous assays, however, revealed that they all had a minimal limit of detection of 75 copies/mL or higher. Molecular assays can differ in their methodologies, molecular target(s), range of quantification and, perhaps most importantly in this case, limits of detection. Given these differences, the manner in which results are reported can be a source of confusion. For results at or below the analytical sensitivity of an assay, some techniques are able to distinguish **1**) no viral nucleic acid detected from **2**) viral nucleic acid is detected but cannot be measured precisely. In this situation, a result of <75 copies/mL would mean that the virus is present at very low levels. However, other HIV-1 quantitative methods are unable to accurately distinguish the presence or absence of HIV-1 RNA below the limit of detection. With this type of assay, a result of <75 copies/mL could represent either no HIV-1 RNA or <75 copies/mL. Proper interpretation of the results requires knowledge of the assay’s performance characteristics, which should be outlined concisely in the associated clinical report. Newer assays have improved analytical sensitivity and can detect 20–40 copies/mL with a broad linear range (upper limit of detection: 10 million copies/mL).<sup>8</sup>

Another important caveat to consider is that HIV-1 has a high rate of mutation; as such, HIV-1 primer site mutation causing failure of PCR amplification can be another cause of lack of identifiable HIV-1 viral load in a new patient with positive serologic testing. For example, mutations at a single position of a downstream primer, found in two percent of all HIV-1 *M gag* sequences, can cause underestimation of HIV-1 RNA levels by more than 100-fold using a real-time PCR assay.<sup>9</sup> In some populations, assay failure rates as high as four percent have been reported due to variation in the *pol* and *gag* genes,<sup>10</sup> and there are multiple reports of viral load discrepancies when using different platforms.<sup>11,12</sup> Some methods, including the highly sensitive technique used in the presented case, address this concern by interrogating multiple gene targets, although measuring plasma HIV-1 RNA by an alternative assay is recommended in clinical practice when low or undetectable viral loads are observed in seropositive patients, especially those with low CD4 counts.

Other potential causes of negative HIV-1 viral load assays in HIV-1 antibody positive persons include HIV-2 infection, effective ART, HIV-1 type O infections, and falsely positive antibody tests. HIV-1 Western blots can sometimes be falsely positive in HIV-2 infection, and older generation HIV-1 RNA assays may not detect HIV-1 type O infections. Use of a confirmatory antibody assay that detects and distinguishes both HIV-1 and HIV-2 can be useful in such circumstances, as can use of newer-generation HIV-1 RNA assays. HIV-1 antibody tests that are confirmed by a second antibody assay are only exceptionally falsely positive, mandating referral to an expert when HIV-1 viral load tests are negative in such circumstances.

### **Conclusion**

In summary, molecular methods are a powerful technique to detect early HIV-1 infection, provide patient prognostication, and help direct therapy. However, results must be considered in light of the clinical presentation and concurrent serologic testing. Lack of a detectable viral load in a patient positive by serological screening may indicate a false-positive test, an HIV-1 primer site mutation causing failure of PCR amplification, HIV-2 infection, use of an insensitive viral load assay, or an elite controller. Consultation with HIV experts and laboratory personnel may help clarify cases of indeterminate or inconsistent testing. □

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## Test yourself



Here are three questions taken from the case report. Answers are online now at [www.amp.org/casereviews](http://www.amp.org/casereviews) and will be published in CAP TODAY next month.

1. Some individuals with HIV infection by repeated serologic testing have extremely low (<50 copies/mL) or unquantifiable HIV RNA-1 viral loads. Reasons for this discrepancy may include all of the following *except*:
  - a) The patient may be an “elite controller,” having a rare (less than one percent) phenotype that has the same viral control as patients taking ART.
  - b) The patient has a new HIV-1 infection and a low viral load is expected at time of seroconversion.
  - c) The patient has a HIV-1 primer site mutation. Testing by an alternative assay may lead to a detectable viral load.
  - d) The patient may have an HIV-2 infection.
2. A newly diagnosed HIV-1 patient is referred to the clinical laboratory for HIV-1 viral load testing as part of his therapeutic monitoring. What viral load corresponds to suppression of viremia and successful ART therapy?
  - a) 50–200 copies/mL
  - b) <500 copies/mL
  - c) <50 copies/mL
  - d) <1,000 copies/mL
3. Nucleic acid testing for HIV-1 is useful in which of the following clinical situations?
  - a) Monitoring response to ART
  - b) Diagnosing a patient in the serological window period
  - c) Identifying an elite controller
  - d) All of the above

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