Increased Detectability of Plasma HIV-1 RNA after Introduction of a New Assay and Altered Specimen-Processing Procedures

Peter F. Rebeiro,1 Asghar Kheshti,2,4 Sally S. Bebawy,1 Samuel E. Stinnette,1 Husamettin Erdem,1 Yi-Wei Tong,1,2 Timothy R. Sterling,1,3 Stephen P. Raffanti,1,4 and Richard T. D’Aquila1

1Division of Infectious Diseases, Department of Medicine, 2Molecular Infectious Diseases Laboratory, Department of Pathology, and 3Center for Health Services Research, Vanderbilt University School of Medicine, and 4Comprehensive Care Center, Nashville, Tennessee

After changes to assay and specimen-processing methods, plasma human immunodeficiency virus type 1 (HIV-1) RNA was frequently detectable in patients who previously had well-suppressed HIV-1 RNA levels. This artifact is attributable to shipping frozen plasma in primary plasma preparation tubes and is not caused by the HIV-1 RNA detection assay; it can be avoided by shipping plasma in a secondary tube.

HIV-1 RNA quantitation in plasma is fundamental to the administration of antiretroviral therapy (ART) [1]. Characterization of HIV-1 RNA levels as being below the limit of detection indicates ART adherence and effectiveness [2, 3]. Conversely, detectable HIV-1 RNA levels may suggest poor treatment adherence or virological failure. Undetectable HIV-1 RNA in a patient receiving ART is required for organ transplantation or entry in certain clinical trials (such as AIDS Clinical Trials Group trial A5244) [4, 5]. If HIV-1 RNA is detectable during therapy of pregnant HIV-infected women, cesarean section may be considered instead of vaginal delivery [6]. Therefore, the correct detection of HIV-1 RNA level is critical during ART.

Beginning 18 June 2007, the COBAS AmpliPrep/COBAS TaqMan HIV-1 test (Roche) replaced the ultrasensitive AMPLICOR assay, version 1.5 (Roche), and the procedure for specimen processing changed at the Comprehensive Care Center (Nashville, TN). Soon thereafter, clinicians recognized that many patients with long-term, consistently suppressed HIV-1 RNA levels who were receiving stable ART had newly detectable HIV-1 RNA. Systematic review of HIV-1 RNA data suggested an artifact. Assays and specimen processing techniques were compared to determine the cause.

**Patients and methods.** All patients receiving stable ART with >1 test results demonstrating undetectable HIV-1 RNA by the AMPLICOR ultrasensitive assay during the first quarter of 2007 (1 January through 31 March) and any HIV-1 RNA result by the AmpliPrep/TaqMan assay after 18 June 2007 were identified. All clinical assay testing was performed at a single national reference laboratory. The same inclusion criteria were applied to data from 2006. HIV-1 RNA was considered to be undetectable if levels were below the lower limit of quantitation: 50 copies/mL for the AMPLICOR ultrasensitive assay and 48 copies/mL for the AmpliPrep/TaqMan assay. The Vanderbilt University Institutional Review Board approved the study of additional specimens from a repository and from prospective patients. Statistical analyses were performed using STATA, version 10 (StataCorp). Comparisons between groups were performed using Fisher’s exact test.

**Results.** Of 82 patients with plasma HIV-1 RNA previously undetectable with use of the AMPLICOR ultrasensitive assay, 63 (77%) had detectable HIV-1 RNA levels with use of the AmpliPrep/TaqMan assay after 18 June 2007. Among the 63 patients with newly detectable HIV-1 RNA, measurements for 36 (57%) represented a >0.5-log increase, and measurements for 16 (25%) represented a >1-log increase over the detection threshold of the previous assay. This rate of HIV-1 RNA detectability among persons with previously undetectable HIV-1 RNA was higher than that for a group of similarly defined patients tested with only the AMPLICOR ultrasensitive assay during both the first quarter of 2006 and June 2006 (9 [10%] of 94 patients had newly detectable HIV-1 RNA during these time periods), which suggested that some of the detectable specimens tested after 18 June 2007 had increased levels of detection because of an artifact.

The hypothesis that the AmpliPrep/TaqMan assay was more sensitive near the lower limit of detection than the AMPLICOR ultrasensitive assay was tested initially. Paired plasma specimens from the Comprehensive Care Center/Vanderbilt-Meharry Center for AIDS Research Specimen Repository were available for 28 patients with discordant results (HIV-1 RNA levels, 50–1330 copies/mL in the assays performed after 18 June 2007). Both the AmpliPrep/TaqMan and AMPLICOR assays were performed on repository specimens collected during both time periods.
periods (first quarter of 2007 and after 18 June 2007) by the Vanderbilt Molecular Infectious Diseases Laboratory in August 2007 (phase I laboratory testing; figure 1).

With use of the AmpliPrep/TaqMan assay, 4 (14%) of the 28 paired specimens from the first quarter and the period after 18 June 2007 each had detectable HIV-1 RNA levels. The AMPLICOR ultrasensitive assay detected HIV-1 RNA in none of the paired specimens from the first-quarter and 4 (14%) of the specimens obtained after 18 June 2007. There was no significant difference in frequency of detectable HIV-1 RNA between assays \((P = .36)\) or in category changes (undetectable to detectable) by assay type. The AmpliPrep/TaqMan assay yielded detectable results for 8% of those specimens in which HIV-1 RNA was undetectable by use of the AMPLICOR ultrasensitive assay. Of 6 repository specimens obtained after 18 June 2007 that had HIV-1 RNA detectable by either assay, 2 had detectable HIV-1 RNA by both assays (AMPLICOR, 315 and 365 copies/mL; AmpliPrep/TaqMan, 208 and 77 copies/mL), 2 had detectable HIV-1 RNA only by the AMPLICOR assay (65 and 167 copies/mL), and 2 had detectable HIV-1 RNA only by the AmpliPrep/TaqMan assay (50 and 110 copies/mL). The minimally increased sensitivity of the newer assay did not explain the large percentage (77%) of detectable HIV-1 RNA reported from clinical testing after 18 June 2007. Furthermore, 22 (79%) of the 28 specimens that were reported to have detectable HIV-1 RNA levels in routine clinical testing with AmpliPrep/TaqMan (HIV-1 RNA levels, 50–1330 copies/mL) were not confirmed to have detectable HIV-1 RNA levels by either assay in this later quality-assurance comparison. This indicated that the increased proportion of detectable HIV-1 RNA noted after 18 June 2007 was an artifact and was not attributable to the new assay itself.

Specimen-processing methods were evaluated next. All spec-

![Figure 1. Flow charts of 2 hypotheses tested to explain artifactual high frequencies of detectable plasma HIV-1 RNA. Phase I tested the hypothesis that the new COBAS AmpliPrep/COBAS TaqMan HIV-1 RNA detection assay (Roche) was more sensitive than the ultrasensitive AMPLICOR assay, version 1.5 (Roche), that was previously used. Paired specimens (processed differently from specimens sent for clinical testing) from the Comprehensive Care Center/Vanderbilt-Meharry Center for AIDS Research Specimen Repository from 28 of the patients whose specimens had yielded discrepant clinical test results were tested at the Vanderbilt Molecular Infectious Diseases Laboratory (MIDL). Similar percentages of detectable HIV-1 RNA were observed with use of the AMPLICOR and AmpliPrep/TaqMan assays. Phase II aimed to confirm whether the artifact was caused by a change to shipment of a frozen primary plasma preparation tube (PPT). Sixteen new patients were enrolled prospectively, and blood was collected in PPTs. Secondary processing for clinical testing with the AmpliPrep/TaqMan assay included decanting plasma into a separate tube for frozen shipment to a national reference laboratory. In addition, a specimen in a primary PPT was frozen for research and was shipped to MIDL, where it underwent AMPLICOR standard (no virion concentration) and ultrasensitive (virion concentration) testing, as well as AmpliPrep/TaqMan testing. Freezing and thawing of the primary PPT was associated with an artifactual high percentage of detectable HIV-1 RNA with all assays. ACD, acid citrate dextrose; Q1, first quarter.](https://academic.oup.com/cid/article-abstract/47/10/1354/450811)
imens collected for clinical testing from 18 June 2007 through 17 August 2007 had been centrifuged and then shipped frozen in the same plasma preparation tube (PPT; Becton Dickinson Vacutainer System) in which blood was collected (primary PPT). Previously, plasma had been separated into another tube after centrifugation of the PPT and then shipped frozen. The repository specimens tested subsequently had been collected in separate acid citrate dextrose tubes at the same time as the specimen that was sent for initial clinical testing and then stored frozen as aliquots of separated plasma after centrifugation. Laboratory procedures for clinical testing were changed on 17 August 2007 to again require plasma removal from the centrifuged PPT and frozen storage and shipment to the national reference laboratory in a separate secondary tube. After this change, 107 (85%) of the next 126 plasma specimens from patients who had undetectable HIV-1 RNA with use of the AMPLICOR assay in the first half of 2007 had an undetectable result with use of the AmpliPrep/TaqMan assay. We next compared each processing method with use of specimens collected prospectively from 16 patients on stable ART regimens with ≥2 prior undetectable HIV-1 RNA measurements. Specimens were processed 2 ways after collection in PPTs. Centrifugation and frozen plasma shipment in the primary PPT was compared with frozen plasma shipment in a secondary tube. We also tested whether concentration of virions after thawing and before performing the AMPLICOR assay might minimize the artifacts seen in specimens frozen in primary PPTs. Specimens frozen in the primary PPT from 8 (50%) of 16 patients yielded positive results when tested with the AMPLICOR without virion concentration, and 7 (44%) of 16 of these same specimens had positive results from the AMPLICOR test after virion concentration. Nine (56%) of 16 of these primary PPT specimens had positive results from the AmpliPrep/TaqMan assay (without virion concentration) \(P = .934\). All 16 patients had undetectable HIV-1 RNA from clinical testing by AmpliPrep/TaqMan of plasma samples obtained concurrently and shipped in secondary tubes to the reference laboratory, with no statistically significant difference by preparation method or assay (phase II laboratory testing; figure 1).

Discussion. After the introduction of the AmpliPrep/TaqMan assay, 77% of patients with previously undetectable HIV-1 who were receiving stable ART had detectable HIV-1 RNA levels. Comparison with results from the previous year suggested that the increased frequency of detectability was an artifact. Independent quality-assurance testing of paired plasma samples indicated that differences in the assay alone did not cause this increase in HIV-1 RNA detection and implicated frozen plasma storage and shipment in the primary PPT as the cause. An increase in the frequency of detection of HIV-1 RNA was observed for every assay tested with plasma that was frozen and thawed in the primary PPT (AMPLICOR with or without virion centrifugation and AmpliPrep/TaqMan). Transferring plasma from a centrifuged PPT into a secondary tube before freezing and shipment corrected the high proportion of detectable HIV-1 RNA from patients with stably suppressed HIV-1 RNA levels.

Previous studies hypothesized that the freezing and thawing of platelets and other cellular elements leads to “leakage” of HIV nucleic acid across the polyethylene gel barrier that separates cells from the acellular plasma in PPTs after centrifugation [7–11]. One report suggested that concentrating the virions in plasma taken from a primary PPT that has been frozen and thawed prior to HIV-1 RNA assay performance might minimize this leakage [9]; this was based on the hypothesis that “naked” HIV nucleic acid released in the supernatant from cells lysed by freezing and thawing would be discarded after centrifugation. The effect of concentrating the virions in plasma was tested, because additional processing at the laboratory, rather than at the clinic collection site, might be advantageous for some clinics. However, virion concentration did not completely eliminate the artifactual detection of HIV-1 RNA. This suggests that at least some of the HIV-1 nucleic acid released by the freezing and thawing of primary PPTs may be located in structures that pellet on centrifugation (such as HIV virions or cores) or that enough free nucleic acid to be amplified is present in the small volume of residual supernatant that overlays the pellet. Even an artifactual increase in HIV-1 RNA level that is large enough only to reach the lower limit of detection may mean the difference between considering a patient as having a treatment response or a treatment failure; this is problematic for trial end points and has important clinical implications [4–6]. Proper laboratory procedures are essential for minimizing artifacts; these procedures include proper maintenance and decontamination of automated instruments used for sample preparation, amplification, detection, and calculation of results. This study indicates that clinicians should be aware of specimen-preparation procedures and should not send plasma frozen in primary PPTs for HIV-1 RNA detection assays. In addition, testing laboratories should reject primary PPTs received for any HIV-1 RNA assay. Some clinics may not have facilities that allow for plasma to be placed in a separate tube after centrifugation of the primary PPT. In such circumstances, clinicians should refer patients to a laboratory that has the capacity for proper specimen processing after the collection of a blood sample, to avoid spuriously detectable viral loads.

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References