Repeat testing of low-level HIV-1 RNA: assay performance and implementation in clinical trials

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Objective: Assess the performance of HIV-1 RNA repeat testing of stored samples in cases of low-level viremia during clinical trials.

Design: Prospective and retrospective analysis of randomized clinical trial samples and reference standards.

Methods: To evaluate assay variability of the Cobas AmpliPrep/Cobas TaqMan HIV-1 Test, v2.0, three separate sources of samples were utilized: the World Health Organization (WHO) HIV reference standard (assayed using 50 independent measurements at six viral loads <200 copies/ml), retrospective analysis of four to six aliquots of plasma samples from four clinical trial participants, and prospective repeat testing of 120 samples from participants in randomized trials with low-level viremia.

Results: The TaqMan assay on the WHO HIV-1 RNA standards at viral loads <200 copcopies/ml performed within the expected variability according to assay specifications. However, standards with low viral loads of 36 and 18 copies/ml reported values of $\geq$ 50 copies/ml in 66 and 18% of tests, respectively. In participants treated with antiretrovirals who had unexpected viremia of 50–200 copies/ml after achieving <50 copies/ml, retesting of multiple aliquots of stored plasma found <50 copies/ml in nearly all cases upon retesting (14/15; 93%). Repeat testing was prospectively implemented in four clinical trials for all samples with virologic rebound of 50–200 copies/ml ($n = 120$ samples from 92 participants) from which 42% (50/120) had a retest result of less than 50 copies/ml and 58% (70/120) retested $\geq$ 50 copies/ml.

Conclusion: The TaqMan HIV-1 RNA assay shows variability around 50 copies/ml that affects clinical trial results and may impact clinical practice. In participants with a history of viral load suppression, unexpected low-level viremia may be because of assay variability rather than low drug adherence or true virologic failure. Retesting a stored aliquot of the same sample may differentiate between assay variability and virologic failure as the source of viremia. This retesting strategy could save time, money, and anxiety for patients and their providers, as well as decrease follow-up clinic visits without increasing the risk of virologic failure and resistance development.

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Introduction

Virologic success for HIV-1 treatment is the achievement and life-long maintenance of undetectable HIV-1 RNA, often defined as less than 50 copies/ml [1–3]. All biological assays, including quantification of HIV-1 RNA, show variability in the reported results. Although virologic failure is defined by HIV treatment guidelines as confirmed by HIV-1 RNA greater than 200 copies/ml, considerable uncertainty exists about the clinical significance of HIV-1 RNA between 50 copies/ml and 200 copies/ml [3]. In clinical practice, after achieving less than 50 copies/ml, an HIV-1 RNA value $\geq$ 50 copies/ml may trigger a follow-up clinic visit with an additional blood draw, and, if confirmed, more frequent monitoring of HIV-1 RNA or a change in antiretroviral regimen. For people living with HIV-1, a report of being 'detectable' may cause substantial anxiety. In the context of clinical trials of HIV-1 treatment, primary efficacy endpoints for many studies are based on the United States Food and Drug Administration snapshot (US FDA)-defined snapshot algorithm for the last available and often single viral load result obtained in the visit window – generally Week 48 for studies of treatment-naïve participants [4]. In the simplest interpretation of this analysis, there are three possible outcomes: HIV-1 RNA less than 50 copies/ml, $\geq$ 50 copies/ml, and no data in the window. Current trials of HIV-1 result in high efficacy rates and the outcomes of a few participants can influence the interpretation of antiretroviral efficacy. For example, a participant with a viral load of 51 copies/ml is classified as HIV-1 RNA $\geq$ 50 copies/ml (virologic failure), whereas another with 49 copies/ml is classified as less than 50 copies/ml (virologic success). In cases of HIV-1 RNA $\geq$ 50 copies/ml in clinical trials, current practice is to perform an unscheduled retest visit after adherence counseling. If the retest visit viral load is less than 50 copies/ml and is within the visit window, then that last result is considered the final snapshot result.

Our hypothesis was that for some participants who achieved an HIV-1 RNA level less than 50 copies/ml on therapy, subsequent values $\geq$ 50 copies/ml but less than 200 copies/ml are because of assay variation rather than true virologic failure. Repeat viral RNA testing of an aliquot of plasma stored from the original blood draw could prevent additional follow-up clinic visits and blood sampling. This is supported by current FDA guidance that suggests that repeat testing of the same sample with an initial $\geq$ 50 copies/ml result may retest as less than 50 copies/ml [5] and eliminate a virologic failure assessment caused by a variety of reasons, including assay variability. Here, we present an analysis of assay variability using reference standards and clinical trial samples with low-level viremia and report the outcomes of implementing this strategy prospectively in clinical trials.

Methods

All HIV-1 RNA quantification results in this report were obtained with the Cobas AmpliPrep/Cobas TaqMan v2.0 HIV-1 assay [6] (Roche Diagnostics, Indianapolis, Indiana, USA) and were performed at Covance laboratories in Indianapolis, Indiana; Geneva, Switzerland; and Singapore, Malaysia. The manufacturer's lower limit of quantification (LLOQ) and lower limit of detection (LOD) is 20 copies/ml of HIV-1 RNA. The WHO HIV 2nd International Standard (97/650) for HIV-1 RNA quantification [7] was diluted in HIV-1 negative ethylenediaminetetraacetic acid (EDTA) plasma to cover the lower range of viral loads of the assay. At each WHO standard dilution, 50 individual aliquots were frozen and tested on independent assay runs at the Indianapolis site ($n = 20$), Geneva site ($n = 20$), and Singapore site ($n = 10$). The functional sensitivity (LLOQ) was confirmed by the lowest concentration that did not exceed a 20% coefficient of variation (CV; results in $\log_{10}$). The LOD was confirmed by the lowest concentration that showed 95% positivity (HIV-1 RNA detected).

In an initial assessment, four to six aliquots of plasma were obtained from four HIV-1 infected participants (15 samples) enrolled in studies GS-US-141-1475 (NCT02397694) and GS-US-380-1844 (NCT02603120) [8]. Plasma samples were obtained, divided into aliquots, frozen on dry ice, and shipped to Covance Central Laboratories. The first HIV-1 RNA result was obtained during the course of the study followed by later testing of HIV-1 RNA from previously unthawed aliquots tested in parallel. Following the analysis of samples from the initial four participants, 120 additional samples from 92 participants were prospectively assessed during the conduct of clinical studies GS-US-380-1489 (NCT02607930), GS-US-380-1490 (NCT02607956), GS-US-380-1844, and GS-US-380-1878 (NCT02603107) in which repeat testing was performed on previously unthawed samples in cases of HIV-1 RNA 50 – 200 copies/ml after achieving less than 50 copies/ml. Exact confidence interval (CI) was obtained using Blaker's method.

Results and Discussion

To quantify the variability of the TaqMan v2.0 HIV-1 assay at low viral loads, six standards with estimated HIV-1 RNA copy numbers of 142, 71, 36, 18, 9, and 4 copies/ml were generated by dilution of the WHO 2nd HIV International Standard. Fifty aliquots of each sample were analyzed (Fig. 1). Overall, the results fell within the manufacturer's specified LLOQ and LOD and the variability in the results was as expected for a biological assay. At the two highest HIV-1 RNA standard concentrations of 142 and 71 copies/ml, 98% of samples reported HIV-1 RNA values greater than 50 copies/ml.

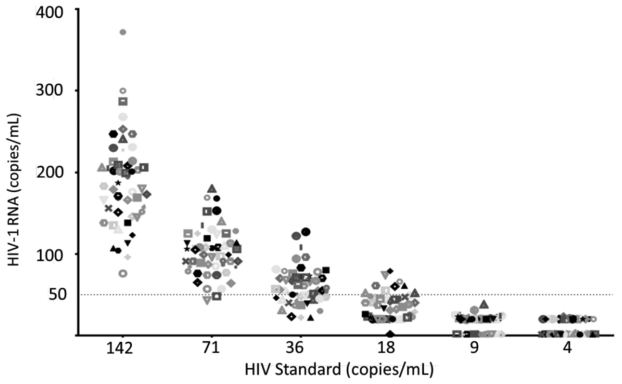

Fig. 1. HIV-1 RNA results of 50 replicates of each dilution of the WHO standard tested at three Covance laboratories by the TaqMan 2.0 assay. The second WHO standard of HIV-1 RNA was diluted and stored in 50 tubes, frozen, shipped as needed on dry ice, assayed, and results from each measurement are plotted for each dilution. The 50 copies/ml threshold for participant management decisions is shown in the gray dotted line. Results were within the expected specifications of the assay. WHO, World Health Organization.

For the 142 copies/ml standard, the mean value was 180 copies/ml (range 76–372 copies/ml). For the 71 copies/ml standard, the mean value was 99 copies/ml (range 43–180 copies/ml). Whenever we tested the standards with estimated HIV-1 RNA levels of 36 and 18 copies/ml, we obtained values of $\geq$ 50 copies/ml in 66 and 18% of the tests, respectively. At the two lowest standard concentrations of 9 and 4 copies/ml, 100% of the samples reported HIV-1 RNA values 50 copies/ml or less.

If applied to clinical samples, a single HIV-1 RNA measurement for participants with virologic rebound and HIV-1 RNA $\geq$ 200 copies/ml or suppression with a low viral load set point of less than 10 copies/ml should provide a clinically correct result (e.g. $\geq$50 copies/ml whenever the real value was greater than 200 copies/ml or less than 50 copies/ml whenever the patient is suppressed). However, if the true HIV-1 RNA value is close to the cut-off of less than 50 copies/ml, a single assay may frequently result as $\geq$ 50 copies/ml because of assay variability and potentially be clinically misleading.

To explore the HIV-1 RNA assay variability near 50 copies/ml in HIV-1 infected and antiretroviral-treated clinical trial participants, we selected four study participants with one or more HIV-1 RNA results less than 50 copies/ml who subsequently had low-level viremia and had additional stored and previously unthawed aliquots of frozen plasma available for repeat testing (Fig. 2a–d). Altogether, in participants having unexpected viremia $\geq$ 50 copies/ml, retesting of multiple aliquots of stored plasma found less than 50 copies/ml in nearly all cases (14/15 aliquots; 93%).

We implemented a repeat testing algorithm for participants with prior suppression of HIV-1 RNA less than 50 copies/ml and virologic rebound between 50 and 200 copies/ml. Specifically, one aliquot of stored plasma was automatically repeat tested by the reference laboratory. The upper limit of 200 copies/ml for this repeat testing was based on the AIDS Clinical Trials Group and the US Department of Health and Human Services guidelines for defining rebound and triggering participant management decisions [3,9,10] and protocol-defined resistance testing if HIV-1 RNA is $\geq$ 200 copies/ml. The samples were required to have been stored frozen and not previously thawed because HIV-1 RNA is stable if properly stored at $-70\,^{\circ}$C but is subject to degradation after either long-term storage or multiple freeze–thaw cycles resulting in incorrect reporting of a lower viral load [11,12].

The prospective analysis had repeat HIV-1 RNA testing performed on 120 sample sets from 92 HIV-1 infected participants from four clinical trials of treatment-naïve or suppressed switch designs (Fig. 2e). Among these sample sets, the initial HIV-1 RNA results consisted of 91 samples with HIV-1 RNA of 50–100 copies/ml and 29 samples with 101–200 copies/ml. The repeat results of HIV-1 RNA less than 50 copies/ml were reported for 50 samples (50/120; 42%; 95% CI 33–51%). Of these 50 samples, the initial HIV-1 RNA was between 50 and 100 copies/ml in all but one sample set (49/50; 98%). Of those samples with a repeat test result $\geq$ 50 copies/ml, 87% (61/70) had follow-up data from subsequent visits and 92% (56/61) resuppressed to HIV-1 RNA less than 50 copies/ml. 5.7% (4/70) of the sample sets were from three participants who later qualified for resistance testing; none had emergent drug resistance.

Overall, the results presented here yield useful information on the variability of results of the Cobas AmpliPrep/Cobas TaqMan v2.0 HIV-1 assay below 200 copies/ml and highlight the challenges of achieving and maintaining a strict clinical endpoint of HIV-1 RNA less than 50 copies/ml. We implemented a repeat HIV-1 RNA-testing protocol utilizing stored samples from the original blood draw and found that more than half (54%; 49/91 samples) of samples with 50–100 copies/ml of HIV-1 RNA repeated as less than 50 copies/ml. These data support the FDA guidance on repeat testing of the same sample to distinguish true virologic failures from other reasons of failure such as assay variability. One limitation of this study is that most participants were taking an antiretroviral regimen consisting of an integrase strands transfer inhibitor with a high barrier to resistance (bictegravir or dolutegravir) in combination with two nucleos(t)ide reverse transcriptase inhibitors, thus reducing its generalizability to other regimens or participant populations. Further discussion of data variability amongst clinicians, people living with HIV, and researchers should lead to guidance on how to best manage low-level rebound.

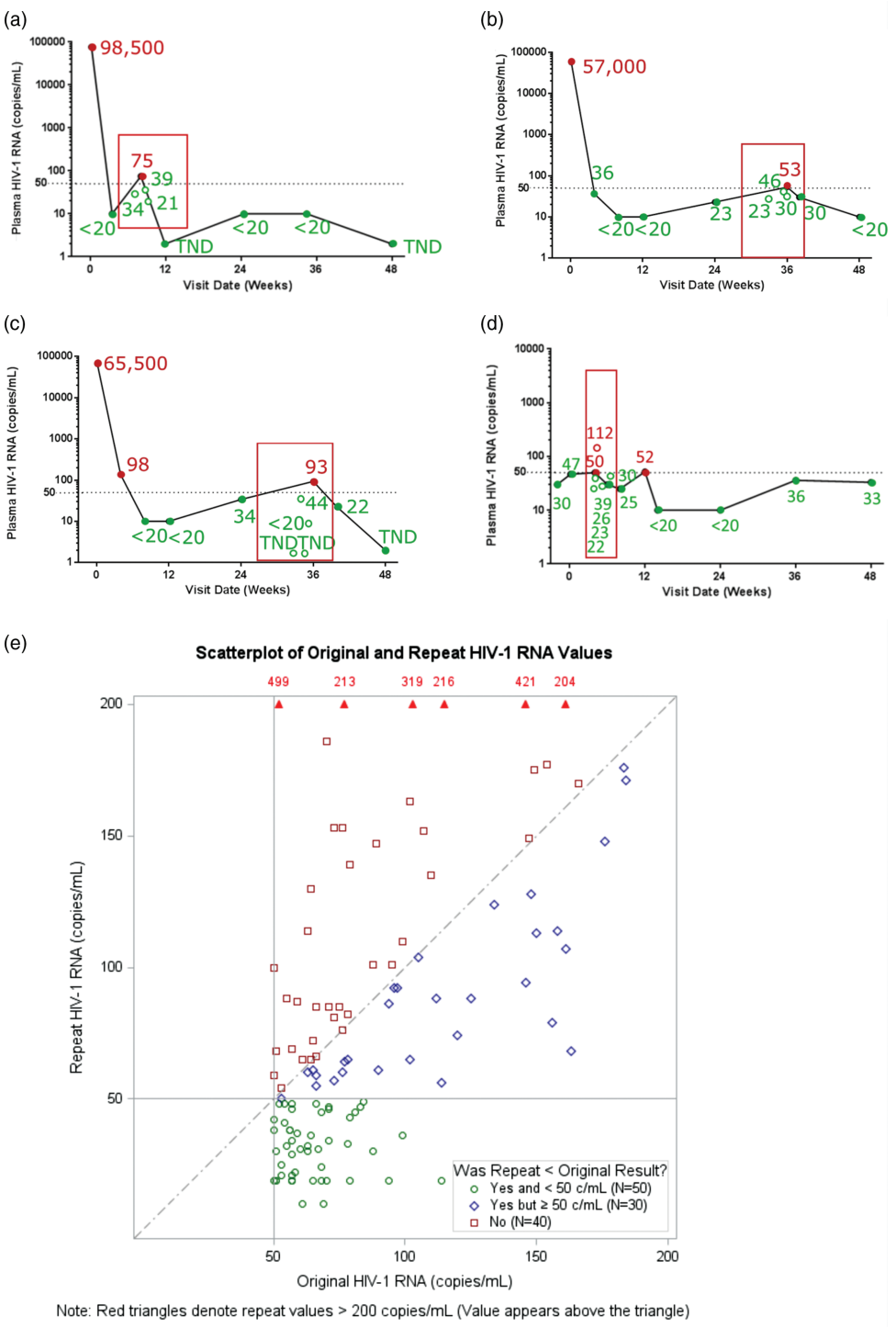

Fig. 2. HIV-1 RNA repeat testing results for participants on antiretroviral therapy by the TaqMan 2.0 assay. HIV-1 RNA tests and retrospective repeat tests from four participants previously naive to antiretroviral therapy (a–c) or suppressed on antiretroviral therapy (d). At baseline and weeks 4, 8, 12, 24, 36, and 48, HIV-1 RNA was measured by TaqMan 2.0. Samples representing viral load blips or suspected blips were retested from stored aliquots. Repeat results are shown as open symbols and original results are shown as filled circles. The clinical cut-off for virologic failure or success is shown at 50 copies/ml (dotted line). (e) Prospective HIV-1 RNA repeat testing of 120 samples. The initial HIV-1 RNA result of 50 – 200 copies/ml (*x*-axis) and repeat test result (*y*-axis). Reference lines at 50 copies/ml for both axes are shown and the dashed line is the 45° line of equality.

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Conflicts of interest

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