Evaluation of Effect of Specimen-Handling Parameters for Plasma Preparation Tubes on Viral Load Measurements Obtained by Using the Abbott RealTime HIV-1 Load Assay

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HIV-1 viral load testing is essential to the management of HIV-1-infected patients, and proper specimen handling ensures accurate viral load (VL) results. This study was performed to (i) evaluate the effect of freezing plasma in situ in BD Vacutainer plasma preparation tubes (PPT) on the accuracy of HIV-1 viral load results using the Abbott RealTime HIV-1 assay and (ii) evaluate the effect of whole-blood storage in the PPT for 6 h at room temperature prior to centrifugation (PPT6H) rather than 2 h as specified in the PPT product insert.

Of the 64 HIV-positive subjects evaluated, 29 had average viral load counts of >40 copies/ml in at least one of the tubes tested and 35 subjects had a result of either “undetected target” or “below the limit of quantification” (LOQ) for all or some of the tubes regardless of handling condition. For the 29 subjects with VLs that were >LOQ, the mean biases between plasma from Vacutainer K2EDTA tubes and plasma frozen in situ in PPT and between K2EDTA tube plasma and plasma from PPT6H (log10 copies/ml) were 0.005 and −0.001, respectively, and r² was >0.92 for all correlations. We conclude that VLs determined from plasma frozen in situ in PPT are equivalent to VLs in K2EDTA tube plasma and can be used for accurate quantification of HIV-1 RNA in the Abbott RealTime HIV-1 assay. Furthermore specimens collected in PPT can be stored for 6 h at room temperature with no effect on viral load results as measured by the Abbott RealTime HIV-1 assay.

Accurate quantification of human immunodeficiency virus type 1 (HIV-1), also referred to as HIV-1 viral load (VL) testing, is essential for effective management of HIV-1-infected patients. The BD Vacutainer plasma preparation tube (PPT) (BD Preanalytical Systems, Franklin Lakes, NJ) was developed to facilitate the handling of plasma specimens used for HIV-1 viral load testing. The PPT contains a K2EDTA additive and a polyester gel which, upon centrifugation, forms a barrier that separates blood cells from plasma, allowing storage, freezing, and shipment of the plasma specimen in situ in PPT. Use of PPT also results in a decrease in the amount of labor required to process specimens, elimination of a potential source of error in specimen labeling, and reduction in the risk of HIV exposure associated with the transfer of separated plasma to secondary tubes for shipping.

Earlier studies using PPT for specimen collection demonstrated compatibility of this tube with HIV-1 VL testing (1, 4, 5). Over the past few years, however, investigators have reported that freezing plasma in situ in a PPT produced higher HIV-1 viral load results compared to plasma from K2EDTA tubes when tested in the Cobas Amplicor HIV-1 monitor system (Roche Molecular Diagnostics, Pleasanton, CA) (2, 3, 11). Elevated levels of HIV-1 VL from PPT frozen in situ after centrifugation were first reported by Squires et al., who showed that PPT yielded higher HIV RNA levels than K2EDTA tubes. The disparity in quantification, seen in both standard and ultrasensitive assays, was more apparent in specimens with VLs between the limit of quantification (LOQ), 50 copies/ml, and 1,000 copies/ml and more pronounced in specimens close to or below 50 copies/ml (3, 10, 11). In fact, studies that compared plasma aspirated from K2EDTA tubes with plasma frozen in situ in PPT show that VLs that are clearly below the LOQ in the former are quantifiable in the latter (3, 8, 11). Such discrepant results for plasma collected in different tubes from the same subject could be interpreted as virological failure, and these results, therefore, have therapeutic implications.

Additional studies reported that specimens which were aspirated and transferred to another tube after centrifugation did not show patterns of artificially increased viral load (8, 10). Similarly, recentrifugation of specimens transported in PPT at 4°C eliminated the inaccurate quantification of HIV seen in plasmas frozen in situ in PPT (6, 9).

Such observations led investigators to associate elevated viral loads in plasma in PPT with the presence of cell-associated nucleic acids released from cells trapped within or adhered to the surface of the gel barrier in PPT (6). Depletion of cellular material by recentrifugation of separated, unfrozen plasma from PPT resulted in lower VLs, indicating that cell-associated nucleic acid contributed to the elevation in VL. This finding was compatible with an earlier study which had shown that the HIV proviral DNA associated with cellular (genomic) DNA was in part responsible for the increase in viral load (13).

The aim of this study was to evaluate the performance of PPT with the new automated RealTime HIV-1 assay (Abbott Molecular, Des Plaines, IL) for the quantification of HIV-1 viral load using the Abbott m2000sp and m2000rt systems for
sample processing and amplification/detection, respectively. Specifically, we were interested in determining the effect of freezing plasma in situ in PPT on HIV-1 viral load analyzed using the Abbott RealTime HIV-1 assay. In addition, the study evaluated the effect on viral load of whole-blood storage in PPT for 6 h before centrifugation rather than 2 h, as specified in the PPT product insert.

MATERIALS AND METHODS

Patient population. All 68 subjects of this study were consenting HIV-positive adults who had previously been shown to have viral load results in the range of 50 to 5,000 copies/ml or below 50 copies/ml within the 60 days prior to enrollment in the study. Study monitors were blind with regard to patient status, CD4 count, drug therapy, sex, and date of diagnosis of infection.

Assay design. A schematic of the study protocol is depicted in Fig. 1. Following hospital procedures for routine venipuncture, blood was collected into four tubes: one 10-ml BD Vacutainer K2EDTA tube (catalog no. 366643) and three 8.5-ml BD Vacutainer plasma preparation tubes (PPT; catalog no. 362799). Tubes were drawn to a randomization schedule, inverted 8 to 10 times immediately after blood collection, and transferred within 2 h postcollection to the laboratory for processing.

On the day of phlebotomy and within 2 h after blood collection, the K2EDTA tube (Fig. 1, tube A) and two PPTs (Fig. 1, tubes B and C) were centrifuged for 10 min at 1,100 x g to separate the plasma. At 6 h after blood collection, a PPT (Fig. 1, tube D) was centrifuged for 10 min at 1,100 x g to separate the plasma. After centrifugation, three 1.1-ml aliquots of plasma were transferred from each of tubes A, B, and D into secondary tubes A1 to A3, B1 to B3, and D1 to D3, respectively, and frozen at −70°C until the day of analysis. The plasma specimen in PPT C was frozen in situ in PPT at −70°C until the day of analysis.

On the day of analysis, plasma aliquots A1 to A3, B1 to B3, and D1 to D3 were thawed, processed, and analyzed for viral load in triplicate using the Abbott RealTime HIV-1 assay. At the same time, PPT C, containing in situ frozen plasma, was thawed and inverted 8 to 10 times and three 1.1-ml aliquots of plasma were transferred into secondary tubes C1 to C3. All plasma aliquots were vortexed three times for 2 to 3 s, centrifuged at 2,000 x g for 5 min, and quantified in the Abbott RealTime HIV-1 assay in accordance with the manufacturer’s instructions.

The average of the three aliquots from each specimen was used as the VL for that tube. If the VL result for an aliquot was “undetected target,” a value of 0 was used to calculate the average. The lower limit of quantification (LOQ) for the assay is 40 copies/ml. In this study, a “detected” result for specimens indicated that the viral load was below the LOQ (<40 copies/ml) but above the “undetected target” level. Viral loads for these specimens were calculated using the assay standard curve (as suggested by the manufacturer) for statistical purposes only.

Statistical analysis. Tubes for which all three aliquots had detectable virus and the average VL was at least 40 copies/ml were used for estimating mean biases among tubes. Analysis of variance (ANOVA)-type models with tube type and subject effects were used to estimate mean biases between PPT B, PPT C, and PPT D and the K2EDTA tube.

The two one-sided test procedure (TOST) was applied to calculate the 95% confidence limits of PPT B, PPT C, and PPT D mean biases versus the K2EDTA tube. Log-log linear regression plots were constructed for comparing PPT B, C, and D to K2EDTA tube A.

Subjects were excluded from the tube bias calculations when the average VL of three aliquots was below the LOQ. For these subjects with very low viral loads, a Cochran Q test was performed and a P value was calculated to determine whether the rates of detection for them were different for the four tubes. Additionally, cross tabulations of tubes with VLs below and above the LOQ were constructed. The exact unconditional McNemar test was used to compare the proportions of subjects with VLs below the LOQ between tubes to determine whether one tube is more likely than another to classify a subject as above or below the LOQ.

RESULTS

A total of 68 subjects participated in the study, of which 64 subjects had complete data sets. Specimens from all 64 subjects were run in triplicate, and the average VL from these three specimens was used for the analysis. Of the 64 subjects, 29 subjects had average VLs that were >LOQ in at least one of the four tube types collected. Thirty-five of these 64 subjects had VLs which were below the LOQ for which the target was undetected in all or most of the tubes collected. The mean biases and correlation coefficients of viral loads between tubes were calculated for the 29 subjects with viral loads that were >LOQ. Data from the remaining 35 subjects were used to determine whether the rate of target detection for these very low viral load subjects would differ depending upon the tube or handling protocol for collection and storage. From all 64 subjects, the data were used to compare the tubes for the proportion of subjects with results that were <LOQ.

Mean bias between tubes. Summary statistics for log_{10} VL for the 29 subjects included in the bias calculations are shown in Table 1. The mean log_{10} viral loads in all four tubes were comparable, as were the medians and the ranges of the VLs in each category. Two-thirds of all specimens in the study had VLs below 1,000 copies/ml. Freezing the plasma in the PPT in situ did not affect the viral load, compared to that for K2EDTA or PPT plasma. Moreover, there was no difference in VL if the specimen was centrifuged and aspirated at 2 h or 6 h postphlebotomy in any of the 87 specimens from the 29 subjects with quantifiable VLs. The mean bias for the log_{10} VL of these subjects for each PPT (tubes B, C, and D) compared to K2EDTA plasma (tube A) is given in Table 2. All comparisons showed that the VLs in each PPT, irrespective of handling conditions, were equivalent to the corresponding VLs in both the K2EDTA tube and the other PPT.

ANOVA-type models with tube type and subject effects
were used to estimate mean biases between PPT (tubes B, C, and D) and the K$_2$EDTA tube (tube A). As seen in Fig. 2, the bias between most tubes was $<0.5$ log$_{10}$ copies/ml and was consistent through the entire study range. As expected, the VL showed more variability for viral load counts of $<100$ copies/ml.

**Correlation of VLs between tubes.** Viral loads in the 29 quantifiable specimens were compared in the log-log linear regression plots for PPT (tube B, C, or D) versus the K$_2$EDTA tube (Fig. 3 to 5). The VL from plasma frozen in situ in PPT correlated strongly to the VL from PPT processed 2 to 6 h postphlebotomy as well as to the VL from the K$_2$EDTA plasma. The $r^2$ values for all comparisons were calculated at $>0.92$. The mean log$_{10}$ differences between the K$_2$EDTA tube and the PPT (tubes B, C, and D) are 0.02, −0.01, and −0.01, respectively, and the agreement limits (±2 standard deviations [SD]) observed in the Bland-Altman plots are ±0.52, ±0.46, and ±0.39, respectively.

**Analysis of VL from specimens below the LOQ.** Thirty-five of the 64 subjects had average viral loads below the LOQ. The data from these 35 subjects are summarized in Table 3. The K$_2$EDTA tube result for 15 of these 35 subjects was “undetected target,” and the corresponding PPT (tube C) had this result for 12 subjects. Similarly, the K$_2$EDTA tube results for 20 subjects and the PPT (tube C) results for 17 subjects were classified as “some aliquots detected.” The four different specimen types did not show significant differences between the numbers of specimens with a result of “detected” or “undetected.” A Cochran Q test, performed to evaluate the rate of detection for these very low VLs, showed a $P$ value of 0.33, indicating no statistical difference among the four tubes. To further examine any variability in the specimens, the exact unconditional McNemar test was used to compare the proportions of subjects with VLs under 40 copies/ml between the four tubes (Table 4). Between-tube agreement was demonstrated for all comparisons, and no significant differences were observed between any tubes. The McNemar tests showed that no tube was more likely than another to classify a subject as above or below the LOQ.

Finally, to determine whether tube type or handling condition had an effect on the distribution of viral loads in the subject population, the frequency distribution of viral loads at different levels was plotted as a function of the tube and handling condition. As seen in Fig. 6, frequency distributions for all four collection conditions were similar.

**TABLE 2.** Estimated mean bias of PPT versus the K$_2$EDTA tube

<table>
<thead>
<tr>
<th>Tube</th>
<th>Bias (log$_{10}$ copies/ml)</th>
<th>95% confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>−0.026</td>
<td>−0.133, 0.081</td>
</tr>
<tr>
<td>C</td>
<td>0.005</td>
<td>−0.101, 0.110</td>
</tr>
<tr>
<td>D</td>
<td>−0.001</td>
<td>−0.107, 0.104</td>
</tr>
</tbody>
</table>

*Tube definitions are as for Table 1.*

![Fig. 2. Scatterplot showing the difference in VLs for PPT (tube B), PPT with plasma frozen in situ (PPT in situ; tube C), and whole-blood storage in PPT for 6 h at room temperature prior to centrifugation (PPT6H; tube D) versus the K$_2$EDTA tube (tube A). Specimens with viral loads below the LOQ show the greatest variability. VLC, VL count.](http://jcm.asm.org/)

![Fig. 3. Log-log linear regression of PPT (tube B) versus the K$_2$EDTA tube (tube A). Correlation coefficients greater than 92% are shown. S, slope; R-Sq, regression coefficient; R-Sq(adj), regression coefficient (adjusted).](http://jcm.asm.org/)

![Fig. 4. Log-log linear regression of PPT in situ (tube C) versus the K$_2$EDTA tube (tube A). Correlation coefficients greater than 92% are shown. S, slope; R-Sq, regression coefficient; R-Sq(adj), regression coefficient (adjusted).](http://jcm.asm.org/)
The current study reports on the effects on viral load results of three handling conditions for HIV-1-positive specimens collected in PPT using the Abbott RealTime HIV-1 viral load assay. In particular, the viral load for each of the test conditions in PPT were compared to values obtained from K2EDTA specimens from the same subject. Our results show that there was virtually no difference in VLs between any of the tubes in the study and that PPT can be used for the determination of VL in the Abbott HIV-1 RealTime assay.

Freezing of plasma in situ in PPT did not result in any differences in the VLs of specimens collected in K2EDTA tubes versus PPT when VLs were quantified in the Abbott HIV-1 RealTime assay and both correlation and agreement results for the K2EDTA tubes and PPT (tube B, C, or D) at lower VLs (40 to 1,000 copies/ml) were excellent. Our observations clearly show that centrifugation of plasma 6 h postphlebotomy does not affect the quantification of HIV RNA in the plasma, as established in our study, that this fully automated method employs a more “RNA-centric” mode of nucleic acid extraction, with minimal contaminating amplifiable proviral DNA. The elevated VLs in PPT measured in the Roche Cobas Ampliprep TaqMan assay appear to be cell associated, indicating that proviral DNA may contribute to the results of HIV quantification. (6, 7). Since VL assays are used as the standard of care for monitoring patients on highly active antiretroviral therapy (HAART), a potentially incorrect result at the time of quantification could have an effect on patient care (7, 11, 12). The accurate quantification of HIV-1 RNA using the Abbott HIV-1 RealTime assay from plasma frozen in situ in PPT, as established in our study, confirms the use of the assay for the reliable measurement of HIV-1 VL.

Table 3. Classification of the 35 subjects with VLs <LOQ

<table>
<thead>
<tr>
<th>Tube</th>
<th>No. (%) of subjects with a result of:</th>
<th>Total no. of subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All aliquots undetected</td>
<td>Some aliquots detected</td>
</tr>
<tr>
<td>A</td>
<td>15 (42.86)</td>
<td>20 (57.14)</td>
</tr>
<tr>
<td>B</td>
<td>12 (35.29)</td>
<td>22 (64.71)</td>
</tr>
<tr>
<td>C</td>
<td>18 (51.43)</td>
<td>17 (48.57)</td>
</tr>
<tr>
<td>D</td>
<td>17 (48.57)</td>
<td>18 (51.43)</td>
</tr>
</tbody>
</table>

a Tube definitions are as for Table 1.

Table 4. Tube comparison with McNemar P values

<table>
<thead>
<tr>
<th>Tubes compared</th>
<th>McNemar P</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/B</td>
<td>.686</td>
</tr>
<tr>
<td>A/C</td>
<td>.532</td>
</tr>
<tr>
<td>A/D</td>
<td>.686</td>
</tr>
<tr>
<td>B/C</td>
<td>.686</td>
</tr>
<tr>
<td>B/D</td>
<td>1.000</td>
</tr>
</tbody>
</table>

a Tube definitions are as for Table 1.

DISCUSSION

The current study reports on the effects on viral load results of the plasma frozen in situ in PPT, suggesting that this proviral DNA, upon amplification in the Roche Cobas monitor assay, yielded quantifiable VL results. Furthermore, since the Amplicor HIV-1 monitor assay employs total nucleic acid extraction in an assay that does not discriminate between amplification of HIV-1 viral RNA and proviral DNA, the presence of cellular, proviral DNA in the plasma is likely to yield elevated VL results.

The Abbott HIV-1 RealTime assay uses automated extraction of viral nucleic acid, which is performed on the m2000sp using purification reagents specific for RNA, followed by amplification with primers and probes that target the integrase region of the HIV-1 genome. The fact that we did not observe elevated VLs in plasma frozen in situ in PPT raises the likelihood that this fully automated method employs a more “RNA-centric” mode of nucleic acid extraction, with minimal contaminating amplifiable proviral DNA. The elevated VLs in PPT measured in the Roche Cobas Ampliprep TaqMan assay appear to be cell associated, indicating that proviral DNA may contribute to the results of HIV quantification. (6, 7). Since VL assays are used as the standard of care for monitoring patients on highly active antiretroviral therapy (HAART), a potentially incorrect result at the time of quantification could have an effect on patient care (7, 11, 12). The accurate quantification of HIV-1 RNA using the Abbott HIV-1 RealTime assay from plasma frozen in situ in PPT, as established in our study, confirms the use of the assay for the reliable measurement of HIV-1 VL.

Table 3. Classification of the 35 subjects with VLs <LOQ

Table 4. Tube comparison with McNemar P values

a Tube definitions are as for Table 1.
regardless of which tube type or handling condition was used for specimen collection and processing.

In conclusion, using the Abbott RealTime HIV-1 viral load assay, specimens can be collected in BD Vacutainer plasma preparation tubes and centrifuged within 6 h of phlebotomy with no effect on viral load results. Furthermore, plasma separated in PPT can be stored frozen in situ until the time of testing with no effect on viral load results.

ACKNOWLEDGMENTS
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REFERENCES


FIG. 6. Distribution of viral load versus tube type for all 68 subjects clearly shows that most of the specimens in the study had viral load counts close to or below the LOQ of the assay.