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In Kenya, HIV-1 viral load monitoring is commonly performed with the Cobas Amplicor using plasma specimens. Interest is growing in transitioning to real-time PCR (RT-PCR), such as the Cobas Ampliprep/Cobas TaqMan (CAP/CTM), using dried blood spots (DBS). Before implementation, direct evaluation of the two assays using DBS field specimens is required. This study compares the sensitivity, specificity, negative and positive predictive values (NPV and PPV, respectively), concordance, and agreement between HIV-1 viral load measurements using plasma and DBS specimens obtained from 512 HIV-1-infected pregnant females enrolled in the Kisumu Breastfeeding Study and tested with the Cobas Amplicor and CAP/CTM assays. The sensitivity and NPV of viral load detection in DBS specimens were higher with CAP/CTM (sensitivity, 100%; 95% confidence interval [CI], 99.1 to 100.0%; NPV, 100%; 95% CI, 99.1 to 100.0%) than the Cobas Amplicor (sensitivity, 96.6%; 95% CI, 94.3 to 98.1%; NPV, 58.8%; 95% CI, 40.7 to 75.4%). The PPVs were comparable between both assays when using DBS. The specificity of viral load detection in DBS specimens was lower with CAP/CTM (77.8%; 95% CI, 40.0 to 97.2%) than that of the Cobas Amplicor (95.2%; 95% CI, 76.2 to 99.9%). Good concordance and agreement were observed when paired plasma and DBS specimens were tested with both assays. Lower specificity with the CAP/CTM is likely due to proviral HIV-1 DNA amplification and lower detection limits with RT-PCR. However, the CAP/CTM has better sensitivity and higher throughput than the Cobas Amplicor. These findings suggest that DBS may be a suitable alternative to plasma when using RT-PCR, which could increase access to viral load monitoring in resource-limited settings.
viral load monitoring (15) and can be performed via two separate procedures: standard or ultrasensitive (16). The linear ranges of the standard and ultrasensitive procedures are 400 to 750,000 copies/ml and 50 to 100,000 copies/ml, respectively (16). Previous reports have established acceptable correlation in HIV-1 viral load detection using the Cobas Amplicor with plasma and DBS (17, 18). In 2007, a real-time PCR (RT-PCR) assay, the Roche Cobas AmpliPrep/Cobas TaqMan HIV-1 test (CAP/CTM) (Roche Molecular Systems, Branchburg, NJ) was introduced (19). The CAP/CTM carries several advantages over the Cobas Amplicor. The CAP/CTM assay is fully automated, uses dual target primers for the Gag and long terminal repeat region, has a lower detection limit (20 copies/ml), has a broader measuring range of 20 copies/ml to 10,000,000 copies/ml, and does not require retesting dilutions of specimens with very high viral loads (20). The CAP/CTM also facilitates efficient processing and high throughput of specimens to allow laboratories to rapidly quantify HIV load in large numbers of specimens (21). A high degree of correlation in HIV-1 viral load measurement between plasma and DBS specimens has been reported using the CAP/CTM assay (22).

The CAP/CTM has not been widely adopted in Kenya or across sub-Saharan Africa, with the exception of South Africa (23). At present in Kenya, very few laboratories utilize CAP/CTM for viral load monitoring of patients enrolled in ART programs. However, given the versatility and ease of use, clinicians and ART program and policy planners are considering the CAP/CTM for routine viral load monitoring, particularly given recent news announcing the discontinuation of the Cobas Amplicor (24). However, prior to implementation, a direct evaluation comparing HIV-1 viral load detection using DBS specimens between the Cobas Amplicor and CAP/CTM is necessary. To our knowledge, no such comparison has been published in the literature. We conducted a study to compare HIV-1 viral load quantification in DBS specimens versus that in plasma using the CAP/CTM, version 2.0, and Cobas Amplicor assays. Specifically, we examined the sensitivity, specificity, negative and positive predictive values (NPV and PPV, respectively), agreement, and concordance of CAP/CTM to the traditional gold standard assay (Cobas Amplicor) on DBS versus plasma specimens as the gold standard specimen type. The applicability of these findings in Kenya and similar resource-limited settings is discussed.

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MATERIALS AND METHODS

Ethical approvals and informed consent. All testing followed approval from the Kenya Medical Research Institute (KEMRI) Scientific Steering and Ethical Review Committees in Nairobi, Kenya, and the Centers for Disease Control and Prevention (CDC) Institutional Review Board in Atlanta, GA. All study participants provided written informed consent for study participation. Consent allowed for storage and future analysis on stored specimens.

Study participant description. The specimens used for this study were collected from participants in the Kisumu Breastfeeding Study (KiBS). The KiBS study design and primary outcomes have been described elsewhere (25–27). Briefly, KiBS was a phase Ib, open-label clinical trial in Kisumu, Kenya, sponsored by CDC, KEMRI, and the Kenya Ministry of Health and intended to evaluate the efficacy of differing ART regimens in preventing mother-to-child HIV transmission (25–27). Patients were enrolled into the study between July 2003 and November 2006. At enrollment, all participating pregnant women were HIV-1 positive and denied a history of receiving ART. Initial HIV infection was determined by a parallel testing algorithm using the Determine HIV-1/2 (Abbott Laboratories, United Kingdom) and the Uni-gold HIV test (Trinity Biotech, Ireland), followed by the Capillus HIV-1/2 (Trinity Biotech, Ireland) for discordant results between the first two assays.

Specimen collection and storage. Upon enrollment in the study, whole-blood specimens were collected from each patient in EDTA anticoagulant tubes. From each whole-blood specimen, paired plasma and DBS specimens were prepared. Plasma specimens were separated from these whole-blood specimens within 6 h from the time of collection and aliquoted and stored at −70°C. Dried blood spots were prepared for each specimen by spotting 75 μl of whole blood on each circle of Whatman 903 filter paper (Whatman, GmbH, Dassel, Germany). The DBS cards were placed on a drying rack and left to dry overnight in a biosafety cabinet at room temperature (20°C). After drying, each card was placed in a glycerine envelope. The glycerine envelopes were subsequently packaged in a zip-lock plastic bag containing four desiccants and a humidity indicator card and stored at −20°C from the time of collection (2003 to 2006) until testing was conducted. During the KiBS, plasma specimens were tested with the Cobas Amplicor as patients were enrolled between July 2003 and November 2006 (26). The remaining plasma specimens were stored at −70°C, and DBS specimens were stored and tested (plasma and DBS with CAP/CTM and DBS with Cobas Amplicor) for the present analysis from January to June 2011. For the present analysis, only specimens from HIV-1-infected pregnant females denying a history of ART enrollment were included.

Nucleic acid processing and detection. When both were available, plasma and DBS specimens were tested with both the Cobas Amplicor and CAP/CTM, version 2.0.

Processing and detection with the Cobas Amplicor, version 1.5. The Cobas analyzer (Roche Diagnostics, Ltd., Rotkreuz, Switzerland) was used for automated amplification and detection. Extraction, amplification, and detection for plasma specimens followed the Cobas Amplicor manufacturer’s standard preparation guidelines. Briefly, 200 μl of plasma specimen was added to 600 μl of working lysis buffer containing the Roche quantitation standard. The subsequent specimen was vortexed and incubated at room temperature for 10 to 15 min. Nucleic acid was precipitated by adding 800 μl of isopropanol and then centrifuged for 15 min at 15,500 rpm (Eppendorf 5417R; Eppendorf, Hamburg, Germany). The supernatant was removed, and 1 ml of 70% ethanol was added to the subsequent pellet for washing, followed by centrifugation at 15,500 rpm for 15 min. Following supernatant removal, 400 μl of diluent was added to dissolve the pelleted nucleic acid. One-quarter (50 μl) of the nucleic acid preparation was added to 50 μl of Roche Amplicor master mix and subjected to amplification and detection using the preset manufacturer’s conditions.

Extraction, amplification, and detection of DBS specimens were identical to those of plasma specimens, with the following modifications. One full circle from each filter card was placed in a specimen QAamp spin column (Qiagen, GmbH, Hilden, Germany) with 600 μl of elution buffer and set for 10 min at room temperature followed by vortexing at 1,000 rpm for 15 min at 56°C. The filtrate was collected, and the eluted material was subjected to the identical extraction, amplification, and detection methods as the plasma specimens, with an exception that the extracts were resuspended in 100 μl of specimen diluent (28).

Processing and detection with the Cobas AmpliPrep/Cobas TaqMan, version 2.0. The Cobas AmpliPrep/Cobas TaqMan 48 analyzer (Roche Diagnostics, Ltd., Rotkreuz, Switzerland) was used for automated extraction, amplification, and quantification following the Roche manufacturer’s standard guidelines. The methodology has been previously published (20, 29) and is briefly described below.

For plasma specimens, 1,100 μl was aliquoted into the respective specimen tubes after vortexing for 5 s. The specimens were transferred to the CAP/CTM analyzer for processing using the H12CAP96 method. For DBS extraction, amplification and quantitation were identical to those of
plasma specimens, with the following modifications. One full DBS circle from each specimen was placed in a 1.8-ml specimen tube. To this tube, 1,000/μl of specimen preextraction reagent was added and the entire specimen was transferred to a Thermomixer (Eppendorf AG, Hamburg, Germany) in which the specimen was continuously shaken at 1,000 rpm for 10 min at 56°C. Extraction, amplification, and viral load quantification of DBS specimens were performed using the CAP/CTM HIV-1 test dried-fluid-spot procedure.

Statistical analysis. All HIV-1 RNA levels in both plasma and DBS specimens were transformed to log10 values. A hematocrit correction calculation, using a population mean hematocrit, was used to determine the concentration of HIV-1 RNA copies in each DBS specimen (28, 30). The detection rates for DBS were calculated for each assay (Table 1). Mean viral load measurements and standard deviations were calculated for each specimen type and assay. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and 95% confidence intervals (CI) were calculated for DBS specimens with each assay by comparing the results to those from plasma specimens tested with the same assay (Table 2). Additionally, precision and agreement of viral load measurements in plasma and DBS specimens with the CAP/CTM and in DBS specimens with the Cobas Amplicor were determined by performing concordance correlation and Bland-Altman analyses with the Cobas Amplicor plasma specimen group as the reference group.

Additional analyses specifically compared viral load quantifications in DBS specimens on the two different platforms. Concordance and agreement of viral load measurements using DBS specimens were compared between the two assay types (CAP/CTM and Cobas Amplicor). Finally, analyses were conducted comparing the viral load quantifications in DBS specimens and plasma specimens as tested with the CAP/CTM. This was intended to evaluate the extent of any differences in viral load results when testing DBS with CAP/CTM compared with plasma. For measurement of concordance, a concordance correlation coefficient and $r^2$ value were calculated. All statistical analyses were conducted using SAS, version 9.2 (SAS, Inc., Cary, NC).

RESULTS

Figure 1 describes the flowchart for specimen inclusion and exclusion in this analysis. From July 2003 to November 2006, 522 HIV-infected pregnant females were enrolled in the KiBS study. Of these 522 patients, 10 patients did not have a blood specimen collected at time of enrollment, and thus plasma and DBS specimens were not available for testing. For an additional 20 patients, plasma specimens were adequate for Cobas Amplicor testing, but insufficient for CAP/CTM. Plasma specimens were available for 512 and 492 patients for Cobas Amplicor and CAP/CTM testing, respectively.

DBS specimens from 445 patients and 476 patients were available for testing with the Cobas Amplicor and CAP/CTM, respectively. Ten specimens had consistently inhibited results with CAP/CTM testing and were excluded from the statistical analyses, leaving 466 DBS specimens tested with the CAP/CTM for inclusion in the analyses.
statistical analyses. A total of 427 plasma and DBS paired specimens were available for testing with the Cobas Amplicor, while 421 pairs were available for CAP/CTM testing.

Paired plasma and DBS specimens tested with each assay were categorized according to ranges of HIV-1 RNA levels measured in the plasma specimens (Table 1). For the Cobas Amplicor group, the ranges, expressed as log$_{10}$ copies/ml, were as follows: undetectable, 2.6 to 2.99, 3.0 to 3.99, 4.0 to 4.99, and >5.0. Among the CAP/CTM group, these ranges, expressed as log$_{10}$ copies/ml, were as follows: undetectable, 1.3 to 2.59, 2.6 to 2.99, 3.0 to 3.99, 4.0 to 4.99, and >5.0. At lower plasma viral loads (<4.0 log$_{10}$ copies/ml), a smaller proportion of corresponding DBS specimens had a detectable viral load when tested with the Cobas Amplicor (Table 1). With the CAP/CTM, all plasma specimens with detectable viral loads had corresponding DBS specimens with detectable viral loads (Table 1). For both assays, two DBS specimens had detectable viral loads, despite undetectable viral loads in corresponding plasma specimens.

The sensitivity of viral load detection in DBS specimens was lower with the Cobas Amplicor (96.6%; 95% CI, 94.3 to 98.1%) than the CAP/CTM (100%; 95% CI, 99.1 to 100.0%). With the Cobas Amplicor, the NPV when using DBS specimens (58.8%; 95% CI, 40.7 to 75.4%) was lower than with the CAP/CTM (100%; 95% CI, 59.0 to 100.0%). The PPVs of viral load detection in DBS specimens were comparable between both assays. The specificity of viral load detection in DBS specimens was higher when using the Cobas Amplicor (95.2%; 95% CI, 76.2 to 99.9%) than the CAP/CTM (77.8%; 95% CI, 40 to 97.2%) (Table 2).

Concordance correlation and Bland-Altman agreement analyses were conducted by comparing a reference group (plasma specimens tested with the Cobas Amplicor) with three comparison groups (DBS specimens tested with the Cobas Amplicor, plasma specimens tested with the CAP/CTM, and DBS specimens tested with the CAP/CTM). The correlation between viral load values obtained from the paired plasma and DBS specimens tested with the Cobas Amplicor assay was strong (Pearson correlation coefficient, 0.8; P < 0.01; concordance correlation coefficient, 0.7; 95% CI, 0.7 to 0.8 for detectable pairs only). The mean log$_{10}$ viral load values were 4.7 copies/ml (95% CI, 4.6 to 4.7 copies/ml) for plasma specimens and 4.3 copies/ml (95% CI, 4.2 to 4.4 copies/ml) for DBS specimens. Thirteen of the 392 paired specimens were below 2 standard deviations from the mean difference between log$_{10}$ viral load values, and none was above 2 standard deviations from the mean difference (Fig. 2a and 3a).

When testing paired plasma specimens with the Cobas Amplicor and CAP/CTM assays, a strong correlation between viral load measurements was observed (Pearson correlation coefficient, 0.8; P < 0.01; concordance correlation coefficient, 0.7; 95% CI, 0.7 to 0.8 for detectable pairs only). The mean log$_{10}$ viral load values using the Cobas Amplicor and the CAP/CTM were 4.6 (95% CI, 4.5 to 4.6 copies/ml) and 4.4 (95% CI, 4.3 to 4.4 copies/ml) for DBS specimens. Twenty of the 431 paired specimens were below 2 standard deviations from the mean difference between log$_{10}$ viral load values, and 3 of the 431 paired specimens were above 2 standard deviations from the mean difference (Fig. 2b and 3b).

Good correlation was observed between viral load values ob-
FIG 2 Concordance correlation analyses of HIV-1 viral load quantification among plasma and DBS specimens collected from patients enrolled in the Kisumu Breastfeeding Study and tested with Cobas Amplicor and CAP/CTM. Cobas Amplicor plasma viral loads were used as a reference group for comparison versus Cobas Amplicor DBS viral loads (a), versus CAP/CTM plasma viral loads (b), and versus CAP/CTM DBS viral loads (c).
FIG 3 Bland-Altman analyses to evaluate agreement in HIV-1 viral load quantification among plasma and DBS specimens collected from patients enrolled in the Kisumu Breastfeeding Study and tested with Cobas Amplicor and CAP/CTM. Cobas Amplicor plasma viral loads were used as a reference group for comparison. The differences between the reference and the comparison assay/specimen type were plotted against the average of the reference group and the comparison assay/specimen type: Cobas Amplicor DBS viral loads (a), CAP/CTM plasma viral loads (b), or CAP/CTM DBS viral loads (c). cp, copies.
tained from the paired plasma and DBS specimens tested with the Cobas Amplicor and viral load values obtained from paired plasma and DBS specimens tested by CAP/CTM (Pearson correlation coefficient, 0.8; P < 0.01; concordance correlation coefficient, 0.8; 95% CI, 0.8 to 0.9 for detectable pairs only). The mean log_{10} viral load values using Cobas Amplicor on plasma and CAP/CTM DBS specimens were 4.6 (95% CI, 4.6 to 4.7 copies/ml) and 4.5 (95% CI, 4.5 to 4.6 copies/ml) copies/ml, respectively. Twelve of the 396 paired specimens were below 2 standard deviations from the mean difference between log_{10} viral load values, and 6 were above 2 standard deviations from the mean difference (Fig. 2c and 3c).

Another analysis compared paired DBS specimens tested with each assay, rather than using the reference group of plasma specimens tested with the Cobas Amplicor. The correlation between viral load values obtained from the paired DBS specimens tested with the Cobas Amplicor and CAP/CTM was strong (Pearson correlation coefficient, 0.9; P < 0.01; concordance correlation coefficient, 0.8; 95% CI, 0.7 to 0.8 for detectable pairs only). The mean log_{10} viral load values using the COBAS Amplicor and CAP/CTM with DBS specimens were 4.3 (95% CI, 4.2 to 4.4 copies/ml) and 4.5 (95% CI, 4.5 to 4.6 copies/ml) copies/ml, respectively. Nine of the 386 paired specimens were below 2 standard deviations from the mean difference between log_{10} viral load values, and 9 were above 2 standard deviations from the mean difference (Fig. 4 and 5).

When comparing viral load values from paired plasma and DBS specimens tested with CAP/CTM, a strong correlation was observed (Pearson correlation coefficient, 0.9; P < 0.01; concordance correlation coefficient, 0.8; 95% CI, 0.8 to 0.9 for detectable pairs only). The mean log_{10} viral load values were 4.4 (95% CI, 4.3 to 4.5 copies/ml) for plasma specimens and 4.5 (95% CI, 4.5 to 4.6 copies/ml) copies/ml for DBS specimens. Eleven of the 401 paired specimens were below 2 standard deviations from the mean difference between log_{10} viral load values, and 9 were above 2 standard deviations from the mean difference (Fig. 6 and 7).

DISCUSSION

For optimum patient care, and given recent findings suggesting early initiation of ART reduces heterosexual HIV transmission, monitoring patients for viral load suppression is of particular importance (8). Additionally, rapid scale-up and increased access to ART in sub-Saharan Africa have made monitoring treatment efficacy and response a rapidly growing need in the region (31, 32). Viral load monitoring still presents substantial collection, storage, and transport-related challenges, some of which usage of DBS could overcome (33, 34). Given the advantages of RT-PCR, assays based on this technology may become the standard of care for viral load monitoring in resource-limited settings. The results of this study suggest that DBS specimens tested with the CAP/CTM have a strong concordance and agreement compared with specimens tested with the Cobas Amplicor, although quantified viral loads

FIG 4 Concordance correlation analysis of HIV-1 viral load quantification among DBS specimens collected from patients enrolled in the KiBS and tested with Cobas Amplicor and CAP/CTM.
were slightly higher with the CAP/CTM. These results demonstrate a wider linear range of detection, especially at lower viral loads, when using DBS specimens with the CAP/CTM in comparison to the Cobas Amplicor. Additionally, the CAP/CTM when using DBS specimens has superior sensitivity and NPV to the Cobas Amplicor. The lower NPV in the Cobas Amplicor could be attributed to lower detection rates in DBS when the plasma HIV RNA copies were lower than 3.0 log10 copies/ml. The lower spec-

FIG 5 Bland-Altman analyses to evaluate agreement in HIV-1 viral load quantification among DBS specimens collected from patients enrolled in the KiBS and tested with Cobas Amplicor and CAP/CTM.

FIG 6 Concordance correlation analysis of HIV-1 viral load quantification among plasma and DBS specimens collected from patients enrolled in the KiBS and tested with CAP/CTM. CAP/CTM plasma viral loads were used as a reference group for comparison versus CAP/CTM DBS viral loads.
ficity of the CAP/CTM when using DBS, however, may require further modifications and additional discussion regarding proper interpretation of viral load measurements performed with real-time assays.

Previous studies have observed that HIV nucleic acids are detectable in whole-blood specimens, even when corresponding plasma specimens have viral load measurements which are undetectable or otherwise below assay cutoff points (35, 36). This may be attributed to amplification of proviral DNA from peripheral blood mononuclear cells (30). Additionally, RT-PCR assays have lower detectable limits, resulting in low-level plasma HIV load detection in comparison to semiautomated or manual techniques (37, 38). The CAP/CTM has previously been demonstrated to result in increased frequency of detectable plasma HIV-1 RNA levels at the threshold of 50 copies/ml among ART patients in comparison with the Cobas Amplicor (39). In the present study, the considerably low specificity of the CAP/CTM with DBS compared to the Cobas Amplicor is likely to be due to a combination of both amplification of cell-associated proviral DNA and increased detection of very low viral loads, which were otherwise undetectable in paired plasma specimens.

Based on the findings of this study, implementation of the CAP/CTM with DBS specimens may result in greater numbers of patients with detectable HIV loads, particularly at low viral loads, than under the current national policies in Kenya, which rely on the Cobas Amplicor using plasma specimens. The clinical implications of this increased detection are unclear. While RT-PCR technologies, including the CAP/CTM, have been shown to result in detection of low-level viral load (20, 37, 39) and increased rates of apparent viral load “blips” (40), these phenomena may not represent subsequent new mutations or treatment failure (37, 38, 41). A recent U.S. government panel recognized that assay variability may result in positive low-level viral loads, with minimal clinical implications (41). Furthermore, WHO guidelines state that the threshold for virological failure in resource-limited settings is >5,000 copies/ml (2). Under both guidelines, the CAP/CTM with DBS specimens, despite low specificity due to amplification of proviral DNA and expanded linear range, may be appropriate for viral load monitoring.

This analysis is subject to the following limitations. First, upon enrollment, the study subjects denied previously receiving ART. The utility to use the CAP/CTM and DBS specimens among patients receiving ART therefore was not assessed. While the findings of this analysis suggest that the CAP/CTM using DBS specimens may be appropriate under the WHO guidelines for detecting viral loads above the threshold of 5,000 copies/ml, further study using specimens from HIV-infected patients during treatment is required. Second, only pregnant HIV-infected females were enrolled in KiBS. To allow for better generalizability of the study findings, similar studies using other patient populations are warranted. Future studies should consider evaluating the effect of HIV proviral DNA and persistent viral load detection on prevention of HIV transmission, a growing area of importance for high-HIV-prevalence regions. Despite these limitations, there was good correlation between DBS and plasma viral loads as well as between the Cobas Amplicor and CAP/CTM. These findings suggest that DBS may be an alternative specimen type to plasma for real-time viral load measurement with the CAP/CTM and could increase access to viral load monitoring in resource-limited settings.

FIG 7 Bland-Altman analyses to evaluate agreement in HIV-1 viral load quantification among plasma and DBS specimens collected from patients enrolled in the KiBS and tested with CAP/CTM.
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Comparison of HIV-1 RNA in Plasma and DBS

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