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Comparison of 25-hydroxyvitamin D concentration in chimpanzee dried blood spots and serum

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Abstract

Background: Dried blood spots (DBS) are used in human medicine to measure total 25-hydroxyvitamin D (25-OHD) in the blood. However, this easy and affordable sampling technique has not been evaluated in primates to measure vitamin D concentrations.

Objectives: We aimed to compare 25-OHD measurements in chimpanzee serum at two different laboratories and determine the precision and accuracy of the DBS method by comparing DBS and serum results.

Methods: Blood samples from 17 captive chimpanzees were collected, and 25-OHD_3 and 25-OHD_2 were measured in serum at two accredited laboratories using liquid chromatography-tandem mass spectrometry. The same analytes were measured on DBS cards, and results were compared with that of serum. Data were assessed using the Spearman correlation, Deming regression, and Bland-Altman analyses.

Results: The correlation coefficient between the two measurements in serum was r_s = .51 (P = .04), and the mean bias was −1.25 ± 14.83. When comparing 25-OHD concentrations measured in DBS and serum at the same laboratory, the r_s was 0.7 (P = .002), and the mean bias was 1.42 ± 14.58. Estimated intra-assay and inter-assay coefficients of variation for DBS results were 6% and 12.6%, respectively.

Conclusions: Although substantial analytical variability was found in 25-OHD measurements regardless of the sample type, the identification of both constant and proportional error and wider limits of agreement with the DBS technique makes the interpretation of DBS results challenging, especially for values close to clinical cut-off points. The DBS and serum methods were not interchangeable, and further studies are needed to validate DBS samples for vitamin D measurements in chimpanzees.

KEYWORDS

great apes, method comparison, primate, vitamin D, Whatman cards

1 | INTRODUCTION

Vitamin D is a fat-soluble vitamin that includes vitamin D_3, produced in the skin from sun exposure, and vitamin D_2, which is obtained from food. In humans and other primates, the predominant circulating form of vitamin D is 25-hydroxyvitamin D (25-OHD), comprising 25-OHD_2 and 25-OHD_3. Although the major function of vitamin D is to maintain adequate serum calcium and phosphorus concentrations in blood, it has a wide range of other biologic actions.[1] Low vitamin D concentrations in people have been associated with both an increased risk of mortality and a variety of disorders, including musculoskeletal diseases, diabetes mellitus, cardiovascular diseases, autoimmune diseases, and cancer.[2,3]

Few studies have assessed vitamin D concentrations in non-human primates[4,5]; however, vitamin D deficiency has been diagnosed in juvenile chimpanzees with rickets,[6] and one study suggested that adult captive chimpanzees experienced vitamin D deficiency when housed without regular access to unfiltered sunlight.[5] Another study measuring nutritional parameters in nine species of captive primates found that most of them had serum 25-OHD levels below published levels for humans and other primates and that chimpanzees had the lowest serum levels.[4] However, since none of these animals showed clinical signs of vitamin D deficiency, the significance of the findings is uncertain, and the question of normal serum vitamin D values in chimpanzees remains unanswered. To establish RIs for this taxon, ideally, concentrations should be measured in healthy individuals living in their natural geographical ranges with unlimited sun exposure. This represents a challenge because of limited access to samples from these populations and little availability of micronutrient analysis in situ.

Precision and reliability of 25-OHD measurements in serum vary depending on the laboratory, analytical technique, and other factors such as equipment calibration and maintenance.[7] The Vitamin D External Quality Assessment Scheme (DEQAS) is the largest specialized external quality assessment scheme for vitamin D metabolites and assessed and monitors the accuracy of results produced by its certified laboratories where 75% of the results have to be within ±25% of a target value.[8] At present, it is accepted that liquid chromatography-tandem mass spectrometry (LC-MS/MS) offers the best accuracy for vitamin D metabolite measurements and is considered the gold-standard technique.[9] However, the DEQAS 2016/2017 review reported that in April 2017, the mean bias of LC-MS/MS assays (against target values assigned by the US National Institute of Standards and Technology) among DEQAS accredited laboratories was 9.3%, which was over the Vitamin D Standardization Program (VDSP) acceptable bias of 5%, and a mean CV of 9.4% (just below the VDSP threshold of 10%).[10-12]

Although serum or plasma samples are the standard biologic specimens used for measuring 25-OHD concentrations in human medicine, it has been shown that 25-OHD concentration measurements in dried blood spots (DBS) are accurate and precise.[13] The DBS technique uses drops of capillary blood collected on filter paper and is minimally invasive and of low-cost, requiring limited sample processing that can be easily applied to field-based research settings. One of the significant advantages of DBS samples is that they do not need to be centrifuged, separated, or frozen following collection. In veterinary medicine, the DBS method has been used for the detection of toxins in mammals and birds,[14,15] pharmacokinetic studies in rats and mice,[16] and avian sexing with PCR.[17] However, this technique has not been validated for any testing in primates.

The objective of this study was to measure 25-OHD (25-OHD_3 + 25-OHD_2) in captive chimpanzee DBSs and calculate the intra- and inter-assay precision and accuracy compared with the measurements in serum. Measurements in serum at two different laboratories were also compared. This project aimed to contribute to the field of zoological medicine by evaluating an easy and affordable sampling technique to measure an important metabolic variable in great apes.

2 | MATERIAL AND METHODS

2.1 | Sample collection

This project has received the approval of the ethical review committee of the University of Nottingham's School of Veterinary Medicine and Science. Routine health-checks were carried out between April and September 2018 on seven male and 10 female chimpanzees (*Pan troglodytes*) held at Twycross Zoo, UK, and that needed to be transferred into a new enclosure. Ages ranged from 11 to 54 years. Premedication consisted of 0.5mg/kg midazolam orally 30 minutes before anesthesia was induced with an intramuscular injection of medetomidine (0.02 mg/kg) and tiletamine-zolazepam (2 mg/kg). Anesthesia was maintained with inhaled isoflurane in oxygen. Respiratory rate, heart rate, body temperature, invasive, and non-invasive blood pressure, end-tidal CO_2, oxygen saturation, and end-tidal isoflurane were monitored continuously. A complete health-check was performed on each animal, including a full physical examination, hematology and biochemistry panels, abdominal and cardiac ultrasound, dental examination, urinalysis, and tuberculosis skin testing. Following standard operating procedures for great ape routine health-checks, 60 mL of venous blood was collected from the femoral vein of all animals for analysis and storage. Three individual DBS cards were prepared for each animal by applying four drops of whole blood directly from the syringe (after removing the needle) onto each card. The cards were provided by the Sandwell and West Birmingham NHS Hospital Clinical Biochemistry department (Whatman 903; GE Healthcare). Considering that each drop of blood collected on the paper card had an estimated volume of 50 µL, it was calculated that approximately 200 µL of whole blood was needed for each DBS card.

2.2 | Sample processing

For serum preparation, whole blood samples were allowed to clot at room temperature for 1-3 hours and then centrifuged (1000*g* for

10 minutes). Serum was separated using a Pasteur pipette and apportioned into 0.5-1.5 mL aliquots. Two 0.5 mL serum aliquots were stored at 7°C overnight and sent to different external laboratories the following day: the Sandwell and West Birmingham NHS Hospital Clinical Biochemistry department ("Laboratory A") and Laboratory Medicine-Central Manchester University Hospitals ("Laboratory B").

At Laboratory B, samples were prepared for analysis by adding 150 µL of an Internal Standard (IS) solution (25-OHD_2-d3 and 25-OHD_3-d6) in acetonitrile to 75 µL of serum, vortex mixed for 5 minutes, and centrifuged for 4 minutes. Thirty microliters of the resultant supernatant were injected into a Transcend II liquid chromatography sample preparation system using TurboFlow online sample preparation technology (Thermo Fisher Scientific). After automated online sample preparation (TurboFlow C8 XL column [0.5 × 50 mm]), the analytes were separated by the analytical column (Accucore C8, [2.6 µm, 2.1 × 50 mm], Thermo Fisher Scientific) maintained at 60°C. The aqueous mobile phase was 10 mmol/L ammonium acetate with 0.1% formic acid in water, and the organic mobile phase (OMP) was 10 mmol/L ammonium acetate with 0.1% formic acid in methanol. Chromatographic separation was achieved with a gradient program of 0-2.33 minutes at 15% OMP; 2.33-3.08 minutes at 80% OMP; 3.08-3.83 minutes with a linear gradient of OMP from 80% to 90%; 3.83-4.83 minutes at 100% OMP; returning to 15% OMP for 4.83-5.83 minutes to re-equilibrate the column. The flow rate was 0.6 mL/min. Mass spectrometric detection was performed using a TSQ Endura tandem quadrupole mass spectrometer (Thermo Fisher Scientific) with atmospheric pressure chemical ionization in positive mode at 400°C. Analyte quantification was determined against Chromsystems Multi-Level Serum 25-OH-Vitamin D3/D2 calibrators (Chromsystems) using TraceFinder (version 3.2) software (Thermo Fisher Scientific). Reported coefficients of repeatability for 25-OHD_3 measurements in human sera were 4.6% at 29.3 nmol/L, and 3.7% at 85.8 nmol/L. Dynamic ranges (quantitative reporting ranges) for the assay were 5-128 nmol/L for 25-OHD_2 and 5-173 nmol/L for 25-OHD_3.

At Laboratory A, liquid-liquid extractions were performed by adding 150 µL of sample, calibrator, or control to 25 µL of the IS solution, and then to 150 µL of a 0.2 mol/L zinc sulfate solution, 300 µL of methanol, and 700 µL of hexane; the mixture was then vortexed and centrifuged. The hexane layer was transferred to a 96-well plate and left to evaporate to dryness, then each well was re-constituted with 80 µL of a 70% methanol:water loading solvent and 20 µL of sample injected onto an Acquity ultraperformance liquid chromatography (UPLC) BEH Phenyl 1.7 µm, 2.1 mm × 50 mm column (Waters Inc.) at a temperature of 35°C.

Chromatographic separation was performed with a similar gradient at Laboratory B with a run time of 4.5 mins at 0.45 mL/min, using electrospray ionization source in positive ion mode. Mass spectrometric detection was performed using a Xevo triple quadrupole (TQD) mass spectrometer (Waters) with a qualifier transition as an added assurance. Chromsystem Multi-Level Serum 25-OH-Vitamin D3/D2 calibrators and Targetlynx data processing software (Waters) were used for quantification. Reported intermediate precisions for 25-OHD_3 measurements in human sera were 7.1% at 27.9 nmol/L and 4.8% at 97.8 nmol/L. Dynamic ranges for this assay were 2.8-450 nmol/L for 25-OHD_2 and 7.5-450 nmol/L for 25-OHD_3.

Both laboratories are accredited by the United Kingdom Accreditation Service, hold an ISO-15189:2012 certification, and participate in DEQAS external quality assurance.[8]

The DBS cards were allowed to dry for 2 hours at room temperature, then placed into hermetically sealed plastic bags containing silica gel and stored at room temperature. Two of the cards for each animal (DBS1 and DBS2) were sent to Laboratory A the day after collection and analyzed within the same batch, and the third card (DBS3) was sent to the same laboratory 1-2 weeks after collection to be analyzed in a different batch. Samples were analyzed using LC-MS/MS on a Waters Acquity UPLC and Waters TQD Mass Spectrometer, following liquid-liquid extraction, as previously described.[18] This human assay has been standardized against human conventional serum and plasma 25-OHD_3 and D_2 LC-MS/MS (Waters TQD MS and Acquity UPLC) using specific blood calibrators (with a standard hematocrit of 45%); hence, blood spot results in people are equivalent to the serum/plasma concentrations if a collected venous sample, without requiring a conversion factor. For the preparation of calibration and quality control samples, raw 25-OHD concentrations in DBS were corrected for the hematocrit fractions using the following formula (values in nmol/L) [18]: $Plasma_{25OHD} = DBS_{25OHD}/(1 - \text{the hematocrit fraction})$. The laboratory-derived intra-assay variation for 25-OHD on human DBS cards is <10% and inter-assay variation was <15%. All DBS samples were analyzed in duplicate by the laboratory, taking a central 3 mm punch from each of the two best blood spots and reporting the results as the average of the two. Dynamic ranges of the DBS assay were 2.8-450 nmol/L for 25-OHD_2 and 7.5-450 nmol/L for 25-OHD_3.

2.3 | Data analysis

As the 25-OHD_2 concentrations were reported to be less than 5 nmol/L in all samples analyzed at Laboratory B and equal to 2.8 nmol/L in all samples analyzed at Laboratory A, only the 25-OHD (vitamin D total) results were included. The mean results for DBS1 and DBS2 pairs were calculated and thereafter called DBS12.

Results were analyzed using GraphPad Prism software (version 8.0.2, La Jolla, CA). The Shapiro-Wilk test was used to test 25-OHD concentrations and calculate the differences (serum lab B-serum lab A, DBS12-serum lab A, DBS3-DBS12) for normality ($\alpha = 0.05$).

Results from serum at Laboratories A and B were analyzed using a non-parametric Spearman correlation and Deming regression, and agreement was evaluated with a Bland-Altman analysis of the differences.[19] Standard deviations used for Deming regressions were estimated from the reported intra-assay variations using the formula SD = CV/100 × mean. One-sample *t* tests were performed on calculated differences (Lab A − Lab B), and linear regression of the differences was plotted on the Bland-Altman graph to detect proportional bias. Outliers were not removed.

TABLE 1 Total 25-OHD concentrations measured in serum and dried blood spot (DBS) cards, calculated differences, and hematocrit results. DBS12 refers to the mean of DBS1 and DBS2

	Serum lab A	Serum lab B	DBS1	DBS2	DBS3	PCV (%)	DBS12	Serum lab A − serum lab B	DBS12 − serum lab A	DBS3 − DBS12	DBS3 − DBS12 (% of DBS12)
	Total 25-OH vitamin D concentrations, in nmol/L										
Chimp 1	81.3	54	76.3	80.6	81.3	41.6	78.45	27.3	−2.85	2.85	3.63
Chimp 2	89.1	70.3	115.7	107.1	81.1	36.2	111.4	18.8	22.3	−30.3	−27.20
Chimp 3	76.3	64	46.4	48.4	51.1	45.8	47.4	12.3	−28.9	3.7	7.81
Chimp 4	81.0	82.8	59.8	55.2	62.9	38.1	57.5	−1.8	−23.5	5.4	9.39
Chimp 5	59.1	59.9	62.5	63.5	52.1	39.9	63	−0.8	3.9	−10.9	−17.30
Chimp 6	69.1	72.8	45.9	49.9	46.1	55	47.9	−3.7	−21.2	−1.8	−3.76
Chimp 7	64.2	72	83.6	75.9	71.2	51	79.75	−7.8	15.55	−8.55	−10.72
Chimp 8	70.2	83.1	51.7	52.2	59.7	42.9	51.95	−12.9	−18.25	7.75	14.92
Chimp 9	72.9	74.8	57.4	63.8	57.5	37.8	60.6	−1.9	−12.3	−3.1	−5.12
Chimp 10	78.7	60.5	71.8	66.5	59.1	32.4	69.15	18.2	−9.55	−10.05	−14.53
Chimp 11	69.7	74.1	60.1	52.6	45.6	60	56.35	−4.4	−13.35	−10.75	−19.08
Chimp 12	66.1	65	46	44.9	52.2	48.3	45.45	1.1	−20.65	6.75	14.85
Chimp 13	124.7	123.7	175.4	157.5	135.3	24.1	166.45	1	41.75	−31.15	−18.71
Chimp 14	122.8	135.1	132.8	125.6	105.2	42.9	129.2	−12.3	6.4	−24	−18.58
Chimp 15	74.3	110.9	100.9	92.6	66.3	28.2	96.75	−36.6	22.45	−30.45	−31.47
Chimp 16	98.9	112.5	106.5	107.7	95	38.3	107.1	−13.6	8.2	−12.1	−11.30
Chimp 17	87.3	91.4	84.7	101.6	78.8	41.2	93.15	−4.1	5.85	−14.35	−15.41

The coefficients of variation and SD for the DBS were calculated using the formulas for the CV and SD based on duplicate samples.[20,21]

Laboratory A (serum Lab A) was considered the reference method, as their DBS technique has been calibrated against a conventional LC-MS/MS on serum. Results from DBS12 and serum Laboratory A were compared and analyzed with the same statistical methods used for the comparison between Laboratories A and B, and acceptability of the DBS method was judged by calculating the percentage of DBS12 results that fall within ±25% of serum Laboratory A results, as this corresponds to DEQAS performance target and has been suggested as the acceptable performance for 25-OHD measurements.[22-24]

Differences between DBS12 and DBS3 results were calculated and analyzed with a one-sample *t* test. Differences expressed in the percentage of DBS12 were calculated and plotted against DBS12.

Finally, the absolute differences between the hematocrit results and a standard hematocrit of 45% were calculated, tested for normality with the Shapiro-Wilk test, and compared with the absolute differences between DBS12 and lab A using a Pearson correlation.

3 | RESULTS

Total 25-OHD concentrations measured in serum and DBS, calculated differences, and hematocrit results, are shown in Table 1.

25-OHD concentrations in serum at Laboratories A and B were not normally distributed, and the Spearman correlation coefficient, r_s, was .5 (95% CI = 0.02-0.8; *P* = .04). Estimated SD at a mean 25-OHD concentration of 82 nmol/L were 3.93 for Laboratory A and 3.00 for Laboratory B. These SD were used to compute a Deming regression, which values were: slope 1.46 (95% CI = 0.66-2.25); *Y*-intercept −35.82 (95% CI = −98.03 to 26.40). Differences between results at the two laboratories were normally distributed. The Bland-Altman analysis revealed a mean bias of −1.25 (SD = 14.83) that did not differ significantly from 0 on a one-sample *t* test (*t* = 0.347, *df* = 16, *P* = .733). On the Bland-Altman plot, magnitudes of the differences did not seem to increase at lower or higher concentrations of 25-OHD: linear regression line slope was −0.28 (95% CI= −0.65 to 0.1), not significantly different from 0 (*P* = .14). The 95% limits of agreements were −30.31 to 27.61. Chimp 15 was identified as an outlier on the Bland-Altman plot with a bias of 36.60 (Figure 1).

The CV for the DBS1 and DBS2 pairs was 6%, and the CV computed between the mean of DBS1 and DBS2 with DBS3 was 12.6%. The calculated SD for the DBS12 pairs was 5.55.

The mean results of the pairs, DBS1 and DBS2 (thereafter called DBS12), were not normally distributed. The Spearman correlation computed between DBS12 and Laboratory A results yielded a correlation coefficient of 0.7 (95% CI = 0.31-0.89; *P* = .024). Values for the Deming regression analysis (using SD = 3.93 for lab A, and SD = 5.55 for DBS12) were slope 1.86 (95% CI = 1.17-2.55); *Y*-intercept −71.41 (95% CI = −125.8 to −17.05). Differences in 25-OHD concentrations between DBS12 and Laboratory A were normally distributed. The Bland-Altman analysis revealed a mean bias of −1.42 (SD = 19.58) that was not significantly different from 0 on a one-sample *t* test (*t* = 0.299, *df* = 16; *P* = .769). 95% limits of agreements were −39.80 to 36.96, with chimpanzee 13 identified as an outlier with a bias of −41.75 (Figure 2). Linear regression analysis of the differences identified a significant positive proportional bias (slope = 0.6; 95% CI = 0.34-0.87; *P* = .0002) and a negative constant bias (*Y*-intercept = −50.30; 95% CI= −72.70 to −27.89), which resulted in an average negative bias at results less than 83.15 nmol/L (*X*-intercept) but with a positive bias above this concentration (Figure 2). Only 9/17 (53%) of the DBS12 results were within ±25% of Laboratory A results (Figure 3).

When comparing results from DBS3 with DBS12, differences were normally distributed, and the mean difference was −9.47 (SD = 13.22, 95% CI = −16.27 to −2.68), which differed significantly

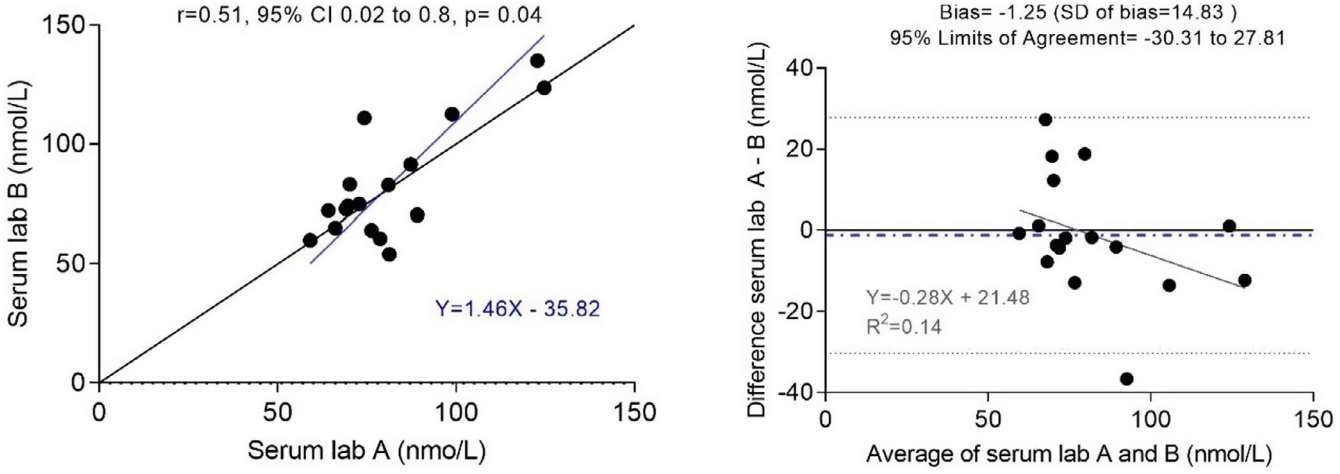

FIGURE 1 Spearman correlation and Bland-Altman analysis assessing agreement between total 25-hydroxyvitamin D measurements in serum at Laboratory A and Laboratory B. Blue continuous line: Deming regression line. Blue dashed line: mean bias. Gray continuous line: linear regression line. Black dotted lines: 95% limits of agreement (±1.96 SD). The identify line (*Y* = *X*) is also provided on the correlation graph

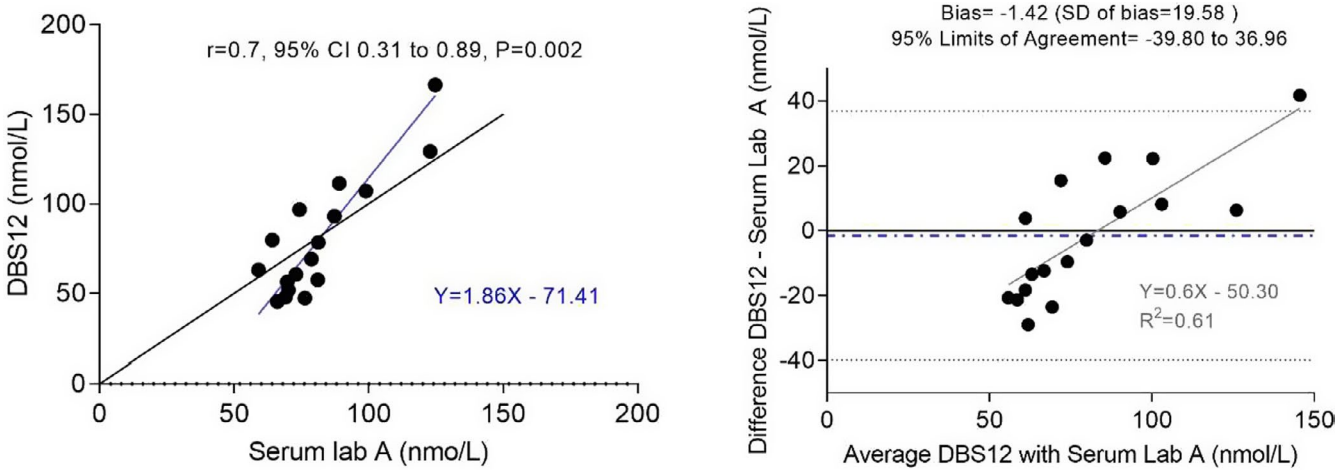

FIGURE 2 Spearman correlation and Bland-Altman analysis assessing agreement between total 25-hydroxyvitamin D measurements in serum at Laboratory A and dried blood spots of the DBS1 and DBS2 pairs (DBS12). Blue continuous line: Deming regression line. Blue dashed line: mean bias. Gray continuous line: linear regression line. Black dotted lines: 95% limits of agreement (±1.96 SD). The identify line (*Y* = *X*) is also provided on the correlation graph

from 0 on a one-sample *t* test (t = 2.96, df = 16; P = .009), showing that the 25-OHD concentration on the DBS3 card was generally lower than on the DBS1 and DBS2 cards. When expressed on a percentage of the DBS12 cards, the differences were between −31.47% and 14.92% (mean difference −8.39% ± 14.21) (Figure 4).

The calculated absolute differences between the hematocrit results and a standard hematocrit of 45% were normally distributed. A Spearman correlation between these differences and the absolute differences between DBS12 and lab A was not significant (α = 0.05) with an r = .11 (95% CI = −0.40 to 0.57; P = .67).

4 | DISCUSSION

The correlation between 25-OHD measurements on chimpanzee DBS cards and serum could be considered strong using traditional approaches of interpreting correlation coefficients.[25] However, limits of agreements were wide, and both constant and proportional errors were identified using the DBS method when compared with measurements in serum at the reference laboratory. Only 53% of DBS card results showed an error within acceptance limits used in people. The correlation was moderate between the measurements in serum at the two different laboratories; however, no significant constant nor proportional error was identified.

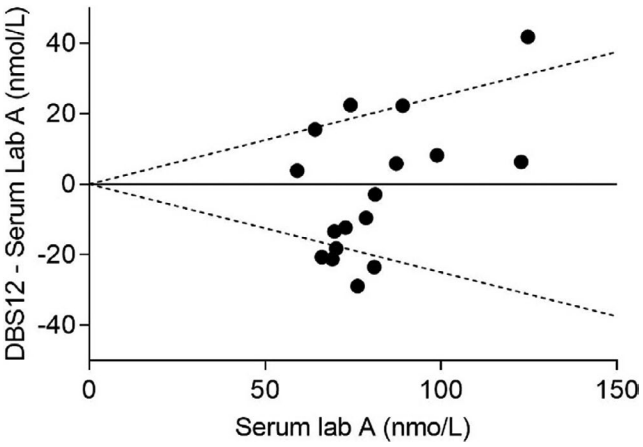

FIGURE 3 Differences between total 25-hydroxyvitamin D measurements in dried blood spots of the DBS1 and DBS2 pairs (DBS12) and serum at Laboratory A showing the ± 25% acceptability lines (black dashed lines)

Liquid chromatography-tandem mass spectrometry is currently established as the gold standard for the measurement of vitamin-D compounds due to improved analytical specificity and sensitivity, and a wider dynamic range compared with immunoassay methods.[9] It is widely recognized that there are substantial differences in human serum 25-OHD measurements not only between assays but also between laboratories using LC-MS/MS.[7] Due to this analytical variability and pre-analytical factors for serum 25-OHD variation (season, sun exposure, and skin type), no evidence-based international consensus on a human vitamin D RI exists.[26] It is, however, recognized that 25-OHD concentrations below 30 nmol/L increase the risk of poor musculoskeletal health, while concentrations between 50-125 nmol/L appear to be safe and sufficient.[27-29] Cases of vitamin D toxicity are rare and associated with serum 25-OHD concentrations above 300 nmol/L.[28]

The presented comparison between 25-OHD measurements in chimpanzee serum at two accredited laboratories allows contextualizing the variation found between serum and the DBS technique. A maximum bias of 36.60 nmol/L and limits of agreements as wide as −30.31 to 27.81 between chimpanzee serum samples stresses the fact that results of vitamin D levels in chimpanzees must be interpreted with care regardless of the sample type, especially for values close to human published cut-off points. For this study, serum samples were sent at ambient temperature as it has been shown that 25(OH)-vitamin D3 in its natural state bound to vitamin D-binding protein is very stable at room temperature,

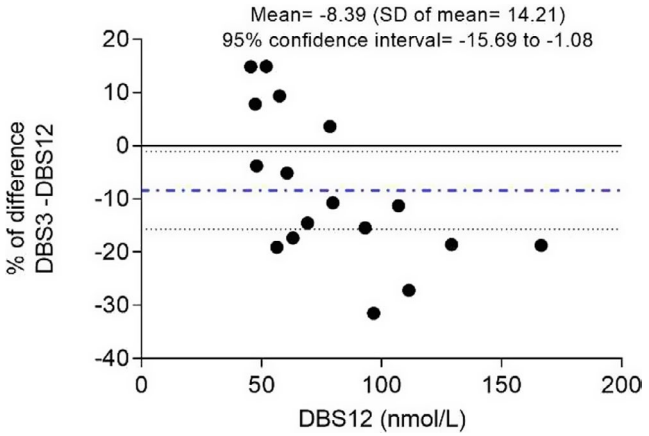

FIGURE 4 Percentage of difference between DBS3 and the DBS1 and DBS2 pairs (DBS12), plotted against DBS12. Blue dashed line: mean % difference. Black dotted lines: 95% confidence interval

and decreases noted after 3 days at around 20°C under common pre-analytical conditions are less than analytical inter-assay precision.[30]

Dried blood spots matrix-matched calibrators used by Laboratory A are produced with a human hematocrit of 45%. As most 25-OHD is present in serum and not in red blood cells, extreme hematocrit values in the analyzed samples could lower the accuracy of the 25OHD results on DBS cards compared with that of serum. The chimpanzees in this study had a wide range of hematocrit levels, ranging from 24.1% to 60%. Interestingly, chimpanzee 13, who had the lowest value of hematocrit, was an outlier in the Bland-Altman analysis comparing serum and DBS results. Although statistical analysis failed to reveal a correlation between the differences in hematocrits (compared with a standard 45%), and differences in vitamin D levels between assays, veterinarians should be aware that vitamin D concentrations measured on DBS cards could be falsely increased in animals with very low hematocrits and falsely decreased in animals with very high hematocrits.

The CV of 6% calculated between the DBS1 and DBS2 pairs encompass both the within-batch analytical CV of the assay for chimpanzees (intra-assay variation) and the variations due to the DBS quality (spot size, diffusion of blood). The result is comparable to the reported intra-assay CV for the human DBS assay of <10%, thus suggesting little variation due to DBS quality in this study. The CV of 12.6% calculated between mean DBS12 and DBS3 additionally incorporates batch assay variation and the stability of vitamin 25-OHD in the DBSs. This result is also comparable to the inter-assay CV of <15% reported by Laboratory A for human DBS. The fact that the third blood spot card, analyzed at a later time point, showed generally lower values than the first two DBSs could put into question the stability of vitamin 25-OHD in a chimpanzee's DBS. However, in unprocessed whole blood, vitamin D metabolites are considered very stable,[30] and a study found no significant effect of different storage condition (at −20°C, 2 weeks in the dark at room temperature, and 2 weeks in full light and open box at room temperature) on vitamin D levels in DBSs.[18] All the DBS cards in this current study were stored in sealed plastic bags with desiccant gel in the dark, to minimize analyte degradation. The difference between the third DBS and the other DBSs could thus be attributable to inter-assay variation only, or to the limited number of samples.

The blood sample used to prepare the DBS in this study was from one single venous blood sample to avoid multiple sampling. DBS cards are designed and calibrated for using capillary blood; however, excellent agreement was found between venous and capillary serum 25OHD concentrations measured by LC/MS-MS in people.[31] A known source of inaccuracy and variation in DBS results come from the quality of the blood spots on the Whitman paper. In a field setting, creating well-sized and homogeneous blood spots is challenging. The laboratory used in this study tried to overcome this problem by performing duplicate measurements using a central 3 mm punch from two of the best spots on the card and providing the mean concentration.

Important study limitations were the relatively low number of samples included and the fact that most of the measured 25-OHD concentrations were in the middle to high part of the analytical range and did not cover the whole working range of the method. This does not adhere to recommendations for method comparison experiments[32]; however, adequate sample numbers and ranges are challenging to achieve when working with zoo species that can only be sampled opportunistically during health-checks or translocations.

Recommendations to calculate the total allowable analytical error for new laboratory methods[32] are difficult to apply in this current study because the inherent imprecisions of the methods used are unknown for chimpanzees, and acceptance limits, based on analytical quality specifications, can only be estimated following recommendations from people. However, as the imprecision estimated for the DBS technique in chimpanzees was similar to the reported imprecisions for human serum and DBSs, the use of acceptance limits based on External Quality Assessment scheme for human 25-OHD seems reasonable. 25-OHD concentrations in serum at Laboratory A were considered as references when estimating the DBS technique acceptability. The fact that only 9/17 of the DBS12 results were within ±25% of laboratory serum results categorizes the DBS technique in chimpanzees as unacceptable according to human quality schemes. However, an important limitation of our study is that the true total-25OHD concentration is unknown; thus, the accuracy of the DBS method in chimpanzees can only be estimated. To validate the DBS method for vitamin D measurements in chimpanzees, further within-laboratory experiments for this sample type are needed, including accuracy measurements against a "true" value, recovery, and interference studies, additional tests on imprecision, and the creation of specific RIs.[33]

Until further validation studies are carried out, zoo and wildlife veterinarians should only use DBS samples to estimate vitamin D status in chimpanzees when serum collection and/or storage is not possible, and avoid making clinical diagnostics based on 25-OHD results from DBS, as the analytical error of this method might not allow discriminating between normal and abnormal values.

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