Original Research

Serum Cobalamin and Folate Concentrations in Common Marmosets (*Callithrix jacchus*) with Chronic Lymphocytic Enteritis

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Serum cobalamin and folate concentrations can serve as surrogate markers of gastrointestinal disease in dogs and cats, where they can have diagnostic, therapeutic, and prognostic implications. Chronic disease of the gastrointestinal tract, particularly chronic lymphocytic enteritis (CLE), occurs frequently in captive common marmosets. The aims of this study were to validate a commercially available assay for measuring serum cobalamin and folate concentrations in common marmosets, to establish reference intervals for these analytes in healthy marmosets, and to measure serum concentrations in common marmosets with CLE. The commercial assay was linear, accurate, precise, and reproducible for the measurement of serum cobalamin and folate concentrations in common marmosets. In healthy marmosets, the serum cobalamin concentration ranged from 322 to 2642 pg/mL ($n = 35$) and serum folate concentration from 54.8 to 786.4 ng/mL ($n = 37$). Low serum folate concentrations were moderately sensitive (greater than 70%) for CLE, and low serum cobalamin concentrations were moderately (greater than 70%) specific for CLE. Both serum cobalamin and folate concentrations were relatively unchanged in marmosets during 120 to 220 d. Serum cobalamin and folate concentrations were stable for approximately 7 y when samples were stored at -80°C . Additional studies are warranted to further study the clinical implications of low serum cobalamin and folate concentrations in common marmosets.

Abbreviations: BMAC, Barshop Marmoset Aging Center; CLE, chronic lymphocytic enteritis; NEPRC, New England Primate Research Center; SNPRC, Southwest National Primate Research Center

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Common marmosets (*Callithrix jacchus*) are a Brazilian New World NHP that has been used in biomedical research since the early 1970s.¹ Common marmosets are a popular species for biomedical research because of their small size (350 to 450 g), relatively low-cost maintenance, easy husbandry, ready habituation to routine clinical procedures, rapid reproductive turnover, and low incidence of zoonoses. Currently, marmosets are used for research in the fields of neuroscience, reproductive biology, infectious diseases, behavior, drug development and safety assessment, and aging research.³⁰

Inflammatory diseases of the intestinal tract, especially inflammatory bowel disease and colitis and particularly chronic lymphocytic enteritis (CLE), have been a consistent finding in several colonies of marmosets.^{10,14,45} Between 1991 and 2000, about 60.5% of all nonexperimental marmosets at the New England Primate Research Center (NEPRC; Boston, MA) had some degree of inflammatory bowel disease.⁴⁴ Similarly, from

2002 to 2011 at the Southwest National Primate Research Center (SNPRC; Texas Biomedical Research Institute, San Antonio, TX), 31% to 44% of marmoset deaths were attributed to inflammatory bowel disease.³⁷ To date, no specific etiology has been proven for this disease in marmosets, but many factors, including gluten sensitivity, dietary protein deficiency, and pancreatic duct parasitism (*Trichospirura leptostoma*), have been speculated to play a causal role.²⁹ CLE is characterized histologically by diffuse to segmental lymphocytic enteritis, and it clinically manifests as failure to thrive in juveniles or weight loss in adults with or without diarrhea.²⁹ Currently, a presumptive antemortem diagnosis is made on the basis of consistent clinical signs, a history of weight loss, and a decreased serum albumin concentration.⁶ Fecal calprotectin,³³ serum matrix metalloproteinases,⁵⁰ and serum IgA antibodies against gliadin and related proteins²⁸ have been measured in marmosets with CLE and may serve as diagnostic markers in the future. To date, no treatment has been shown to be effective in marmosets, and a definitive diagnosis of CLE is usually made at necropsy.²⁹

Cobalamin (vitamin B12) is a water-soluble vitamin that is an essential cofactor for the enzymes methylmalonyl-CoA mutase and methionine synthase. Intestinal uptake of cobalamin is a multistep process, with the absorption of cobalamin–intrinsic factor complexes occurring through specific receptors on ileal enterocytes.^{31,43} Cobalamin deficiency is reported frequently in dogs and cats with exocrine pancreatic insufficiency, distal small

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intestinal disease, or diffuse small intestinal disease and is less commonly attributed to genetic defects in various dog breeds.¹⁹ Exocrine pancreatic insufficiency causes hypcobalaminemia in dogs, in which the majority of intrinsic factor is synthesized by pancreatic acinar cells.^{4,13} Ileal disease is believed to lead to damage or decreased expression of cobalamin receptors, ultimately leading to reduced cobalamin absorption and deficiency once cobalamin body stores have been exhausted. In addition, intestinal dysbiosis can lead to decreased serum cobalamin concentrations, because many bacterial species compete for and bind available cobalamin.²¹ The reported prevalence of cobalamin deficiency in animals with gastrointestinal disease ranges from 6% to 18.5% in dogs^{2,13} and from 17% to 61% in cats.^{35,40} Furthermore, hypcobalaminemia has been associated with negative outcomes in dogs with chronic enteropathy² or exocrine pancreatic insufficiency,³ and cobalamin supplementation may have therapeutic potential.³⁸ Decreased serum cobalamin concentrations have been reported in cats with exocrine pancreatic insufficiency,⁴⁸ inflammatory bowel disease, and gastrointestinal neoplasia such as lymphoma and adenocarcinoma.²⁵ To our knowledge, no data on the cobalamin status of marmosets has been reported to date. However, serum cobalamin concentrations have recently been investigated in rhesus macaques and pigtailed macaques with chronic idiopathic diarrhea.²² Serum cobalamin concentrations were 2.5fold lower in pigtailed macaques with chronic idiopathic diarrhea compared with healthy controls. However, no difference was observed in rhesus macaques.²²

Folate (B9) is another water-soluble B complex vitamin. Dietary folate in the form of folate polyglutamate is poorly absorbed in the intestinal tract. However, the enzyme folate deconjugase in the jejunal brush border converts folate polyglutamate into monoglutamate, which is then absorbed into enterocytes of the proximal small intestine by using specific carriers. Chronic intestinal disease damages folate carriers, reducing folate uptake and thus leading to decreased serum concentrations of folate.⁴² Increased serum folate concentrations are reported in dogs with intestinal dysbiosis, because many bacterial species synthesize folate.²¹ In addition, chronic enteropathy can lead to decreased serum folate concentrations in dogs and cats.^{13,35} To our knowledge, serum folate concentrations have not been evaluated in marmosets with or without gastrointestinal disease.

Dietary deficiency of cobalamin and folate is highly improbable, and it is believed that even withholding food for several weeks does not cause serum cobalamin and folate to become subnormal.⁴² Rather, characterizing the changes in serum concentrations of cobalamin and folate in marmosets with CLE may facilitate monitoring gastrointestinal health in this species. The objectives of the study were to: 1) partially validate commercial immunoassays for measuring cobalamin and folate in human serum for use in common marmosets; 2) establish reference intervals for serum concentrations of cobalamin and folate in healthy common marmosets; and 3) study the changes in the concentration of serum cobalamin and folate in marmosets with CLE.

Materials and Methods

Marmoset serum samples and data. This study was approved by the respective primate centers' IACUC. Serum samples were collected from common marmosets at the Southwest National Primate Research Center (SNPRC), Texas Biomedical Research Institute, San Antonio, TX (Animal Use Protocol no. 1259CJ), the Barshop Marmoset Aging Center (BMAC), and the University

of Texas Health Science Center, San Antonio, TX (protocol no. 06120X). In addition, excess serum from the common marmoset colony at the New England Primate Research Center (NEPRC; Southborough, MA) that was submitted for diagnostic testing at the Gastrointestinal Laboratory (Texas A and M University) was included in this study. Serum samples were transported on dry ice to the Gastrointestinal Laboratory and stored at -80 °C until analysis.

Clinical history and necropsy data from these common marmosets were reviewed to determine whether they were healthy at the time of collection, had clinical signs of gastrointestinal disease, or had histopathologic evidence of CLE.

Analysis of serum cobalamin and folate concentrations. Serum cobalamin and folate concentrations were measured by using automated competitive chemiluminescence enzyme immunoassays (Immulite 2000, Siemens, Hoffman Estates, IL) approved for use in humans. The reportable range of the cobalamin assay is 150 to 1000 pg/mL, and that for the folate assay is 1 to 24 ng/mL. Samples that had results over this reportable range were diluted according to the manufacturer's recommendations by using the sample diluent (catalog no. L2FVZ, Siemens) supplied with the assay kits.

Partial validation. Because validation data for NHP were unavailable, a partial validation for the assays was performed. Because of the small volumes available from individual common marmosets, serum samples were pooled to obtain sufficient volumes; 4 pooled serum samples were used for analytical validation.

Linearity of the assay was determined through dilutional parallelism. The 4 pooled serum samples were serially diluted (1:2, 1:4, 1:8, and 1:16) manually. The observed:expected ratio was calculated and expressed as a percentage. The Immulite analyzer (Siemens) is able to perform sample dilutions during runs, and this process was included during validation. For cobalamin assay validation, we ran 4 serum samples each at dilutions of 1:3, 1:5, 1:10, and 1:20. To validate the folate assay, we ran 5 serum samples each at 1:10, 1:20, 1:40, and 1:100. The analyzer reported the CV as a percentage ($CV\% = SD / \text{mean} \times 100\%$).

The accuracy of the assays was determined by mixing at a 1:1 ratio 3 pooled serum samples with other pooled samples of known concentration. The standard recovery was calculated from the ratio of observed concentration to expected concentration expressed as a percentage). In addition, we added 3 quality-control samples provided with the assay in a 1:1 ratio to 4 serum samples for the cobalamin assay and 5 serum samples for the folate assay; data are reported as the observed:expected ratios calculated as described earlier.

Precision was determined by evaluating 5 replicates each of 4 pooled serum samples during the same run on the same day. Reproducibility was assessed by evaluating 5 replicates each of 4 pooled serum samples during different runs on consecutive days. Both precision and reproducibility were assessed by calculating the CV%, as described earlier.

Stability testing. To assess the stability of cobalamin and folate in serum samples stored at -80 °C, 6 samples analyzed in 2006 were retested in 2013.

Reference intervals. Reference intervals for serum cobalamin and folate were established by using RefVal 2.1 (Department of Clinical Chemistry, Rikshospitalet, Oslo, Norway) adapted for Excel (Microsoft, Redmond, WA) on samples from healthy marmosets at 2 centers (BMAC and SNPRC) with no previous clinical history of gastrointestinal disease.

Additional analysis. Metadata from 2 primate centers (BMAC and SNPRC) were analyzed regarding serum concentrations of cobalamin and folate, specifically assessing location of housing,

Table 1. Parallelism of serum cobalamin concentration (pg/mL) in manually diluted samples from common marmosets

Dilution	Observed concentration	Expected concentration	Observed/expected (%)
Serum 1			
0	485		
1:2	283	242.5	116.7
1:4	167	121.3 ^a	not done
1:8	165	60.6 ^a	not done
Serum 2			
0	391		
1:2	262	195.5	134.0
1:4	188	97.8 ^a	not done
1:8	<150	48.9 ^a	not done
Serum 3			
0	202		
1:2	163	101.0 ^a	not done
1:4	<150	50.5 ^a	not done
1:8	<150	25.3 ^a	not done
Serum 4			
0	456		
1:2	258	228.0	113.2
1:4	182	64.5 ^a	not done
1:8	<150	57.0 ^a	not done

^aThe concentration is below the lower limit of detection of the assay.

animal age (in days) when sample was collected, sex, dam, sire, birthplace, litter size, weaning litter size, adult body weight, obesity status (determined as function of body weight), and age group. Marmosets were categorized into age groups as infants (0 to 5 mo), juveniles (older than 5 to 10 mo), subadults (older than 10 to 15 mo), and young adults (older than 15 mo).⁴⁹

Serum cobalamin and folate concentrations were compared between healthy marmosets and those with any clinical history of gastrointestinal disease according to medical records from these 2 centers. When possible, repeated measurements of serum cobalamin and folate from the same animals were analyzed over multiple time points.

Use of determined reference intervals for analysis. The reference intervals we established were used to assess the concentrations of serum cobalamin and folate of another marmoset colony (NEPRC), where cross-sectional and longitudinal measurements were available. According to diagnosis at necropsy, the marmosets evaluated were categorized as healthy, having CLE, or having another disease as the cause of death. Serum cobalamin and folate concentrations were compared among these 3 groups.

Sensitivity and specificity. Sensitivity and specificity were determined by using an online calculator. Sensitivity was calculated according to the formula:

$$a / (a + b) \times 100\%,$$

and specificity was calculated by using the formula:

$$d / (c + d) \times 100\%,$$

where a is marmosets with CLE and a low serum cobalamin or folate concentration, b is marmosets with CLE and a normal serum cobalamin or folate concentration, c is marmosets without CLE but with a low serum cobalamin or folate concentration, and d is marmosets without CLE and with a normal serum cobalamin and folate concentration.

Table 2. Parallelism of serum cobalamin concentration (pg/mL) in analyzer-diluted samples from common marmosets

Dilution	Observed vitamin B12 concentration	Coefficient of variation (%) between samples
Serum 1		5.8
1:3	1580	
1:5	1392	
1:10	1580	
1:20	not available	
Serum 2		7.0
1:3	1708	
1:5	1445	
1:10	1644	
1:20	not available	
Serum 3		8.8
1:3	1923	
1:5	1728	
1:10	1550	
1:20	not available	
Serum 4		3.8
1:3	1665	
1:5	1538	
1:10	1674	
1:20	not available	

Table 3. Recovery results after 1:1 spiking of serum samples from common marmosets with others of known cobalamin concentrations

	Expected (pg/mL)	Observed (pg/mL)	Observed/expected (%)
Serum 1: Serum 3	340.1	318.0	93.5
Serum 2: Serum 3	293.0	268.0	91.5
Serum 3: Serum 4	325.0	313.0	96.3

Cobalamin concentrations were 486.6, 392.4, 193.6, and 456.4 pg/mL for serum 1, 2, 3, and 4, respectively.

Statistics analysis. Statistical analyses were performed by using commercially available software packages (JMP 10, SAS Institute, Cary, NC; Prism 5.00, GraphPad Software, La Jolla, CA). Significance was defined as a *P* value less than 0.05. Normality was tested by using the Shapiro–Wilk test, which was not met. Where multiple parameters were evaluated, Bonferroni correction was applied. The Wilcoxon signed-rank test was used for repeated measures. Mann–Whitney U and Kruskal–Wallis tests with a Dunn posttest were used to compare between groups.

Results

Validation. Regarding cobalamin measurements, observed:expected ratios (mean $\pm$ 1 SD) for dilutions of 1:1, 1:2, and 1:4 ranged from 113.2% to 282.2% (176.2 $\pm$ 67.3%; Table 1). In addition, CV% were 3.8%, 5.8%, 7.0%, and 8.8% for the 4 serum samples at analyzer-provided dilutions of 1:3, 1:5, and 1:10; at 1:20, the samples were too dilute and therefore failed to give a result (Table 2). The observed:expected ratios for recovery after spiking of 4 samples ranged from 91.5% to 96.3% by using automated dilution (Table 3) and 92.9% to 105.9% (98.2% $\pm$ 5.7%) by using 122.0, 276.0, and 425.5 pg of cobalamin from the quality-control sample provided by the manufacturer (Table 4). The intraassay %CV was 0.4% to 3.4%, and the interassay %CV ranged from 3.5% to 8.4% (Table 5). The manufacturer reports that the analytical sensitivity of the cobalamin assay is 125 pg/mL.

Table 4. Recovery results after 1:1 spiking of serum samples with commercial cobalamin standards

Serum sample	Concentration of spiking solution (pg/mL)	Observed cobalamin concentration (pg/mL)	Expected cobalamin concentration (pg/mL)	Observed/expected (%)	Mean observed/expected (%)
Serum 1	0 (diluent)	297			92.9
	244	374	419	89.3	
	552	513	573	89.5	
	851	722	722.5	99.9	
Serum 2	0 (diluent)	263			95.0
	244	356	385	92.5	
	552	502	539	93.1	
	851	685	688.5	99.5	
Serum 3	0 (diluent)	257			105.9
	244	378	379	99.7	
	552	551	533	103.4	
	851	783	682.5	114.7	
Serum 4	0 (diluent)	278			99.0
	244	388	400	97.0	
	552	570	554	102.9	
	851	683	703.5	97.1	

100 µL of spiking solution was added to 100 µL of serum sample.

Table 5. Precision and reproducibility of serum cobalamin concentration (pg/mL) in common marmosets measured by using a commercial cobalamin assay

Sample	Mean	1 SD	CV (%)
Serum 1	449.8	15.6	3.5
Serum 2	353.4	26.4	7.5
Serum 3	172.8	14.5	8.4
Serum 4	450.2	24.0	5.3
Serum 5	486.6	15.9	3.3
Serum 6	392.4	1.7	0.4
Serum 7	193.6	6.4	3.3
Serum 8	456.4	15.4	3.4

Each sample was tested as 5 replicates.

Regarding folate measurements, observed:expected ratios (mean $\pm$ 1 SD) for the 1:1, 1:2, 1:4, and 1:8 dilutions ranged from 63.0% to 95.0% (73.1% $\pm$ 9.7%; Table 6). The CV% for the concentrations of serum folate were 8.7%, 9.2%, 11.9%, 16.9%, and 20.6% for the 5 serum samples at the dilutions of 1:10, 1:20, 1:40, and 1:100 (Table 7). Observed:expected ratios for recovery after sample spiking ranged from 90.1% to 103.2% (97.0% $\pm$ 6.6%) when serum was used (Table 8) and from 75.0% to 101.4% (85.3% $\pm$ 4.6%) when 1.5, 7.0, or 10.9 ng of folate from the manufacturer's quality control was used (Table 9). The intraassay %CV ranged from 1.8% to 9.4%, and the interassay %CV was 3.7% to 5.9% (Table 10). The manufacturer reports that the analytical sensitivity of the folate assay is 0.8 ng/mL.

No statistically significant differences in the cobalamin and folate concentrations of 6 serum samples stored at -80°C between 2006 and 2013 were identified ($P = 0.58$ and $P = 0.81$ respectively). The median (minimum to maximum) CV% for the cobalamin and folate concentrations of the 6 serum samples analyzed in 2006 and 2013 were 10.5% (2.9% to 21.9%) and 12.5% (2.5% to 22.8%), respectively.

Reference intervals. Serum samples from a total of 53 marmosets (BMAC, 34; SNPRC, 19) were available. Of these animals, 14 marmosets (BMAC, 8; SNPRC, 6) had a history of clinical signs associated with gastrointestinal disease and were excluded from the reference interval calculation. Reasons for exclusion included weight loss ($n = 7$), diagnosis of giardiasis or treatment for *Giardia* spp. ($n = 6$), loose stool ($n = 1$), and transient gastrointestinal upset ($n = 1$).

Because of insufficient sample volume for dilution, final concentrations were not determined for samples from some animals. The reference interval for serum cobalamin concentration was established as 322 to 2642 pg/mL by using samples from 35 healthy marmosets. To achieve the final cobalamin concentrations, serum samples were run undiluted for 18 samples and at a 1:3 dilution for 17 marmosets samples.

The reference interval for serum folate concentration was established as 54.8 to 786.4 ng/mL by using 37 samples from healthy marmosets. To obtain the final concentration, 29 samples were run at a 1:40 dilution, 5 at 1:20, 2 at 1:100 dilutions, and 1 without dilution.

Health comparisons. Sex, dam, sire, birthplace, multiple births, litter size at weaning, obesity status, and age group were not significantly associated with either serum cobalamin or serum folate concentration. Interestingly, serum folate concentrations were significantly ($P = 0.0108$) different between the 2 centers (BMAC, SNPRC) where the samples had been collected. Serum cobalamin concentrations were significantly ($P = 0.0002$) different between healthy marmosets (median, 1020 pg/mL; range, 281 to 2390 pg/mL) and the 14 marmosets with a previous history of gastrointestinal disease (median, 536 pg/mL; range, 150 to 1051 pg/mL). However, folate serum concentrations did not differ between the 2 groups ($P = 0.356$).

Multiple measurements of serum cobalamin and folate concentrations from the same animals were available for at least 2 time points for 30 animals, with 8 marmosets having samples from 3 time points and 1 animal with samples from 4 time

Table 6. Parallelism of serum folate concentration (ng/mL) in manually diluted samples from common marmosets

Dilution	Observed	Expected	Observed / expected (%)
Serum 1			
1:1	13.1	not available	not available
1:2	5.4	6.6	82.3
1:4	2.1	3.3	63.8
1:8	1.1	1.6	64.7
Serum 2			
1:1	17.9	not available	not available
1:2	7.0	9.0	77.9
1:4	2.9	4.5	64.8
1:8	1.4	2.2	63.0
Serum 3			
1:1	9.6	not available	not available
1:2	3.4	4.8	70.6
1:4	1.6	2.4	66.9
1:8	<1.0 ^a	1.2	
Serum 4			
1:1	20.6	not available	not available
1:2	9.8	10.3	95.0
1:4	4.2	5.2	81.7
1:8	1.9	2.6	73.4

^aThe concentration is below the assay's lower limit of detection.

points. Due to insufficient volume, cobalamin measurements could not be performed for 6 serum samples, and folate measurements could not be performed for 2 serum samples. In 23 animals, repeat serum samples for cobalamin concentration were available after a median of 120 d (minimum, 77 d; maximum, 165 d) of the first collection, and results were not significantly different from baseline measurements ($P = 0.726$). In 8 marmosets, serum samples for cobalamin concentration measurements were available after a median of 223 d (minimum, 222 d; maximum, 258), and results were not significantly different from baseline measurements ($P = 0.547$).

In 26 animals, repeat serum samples for the measurement of folate concentration were available after a median of 123 d (minimum, 88 d; maximum, 165 d) of the first collection, and results were not significantly different from the first measurement ($P = 1$). In 7 animals, serum samples for the measurement of folate concentration were available at a median of 223 d (minimum, 222; maximum, 258 d) after baseline collection, and results were not significantly different from baseline data ($P = 0.375$).

Information regarding serum cobalamin and folate concentrations and paired necropsy diagnosis was available for 38 marmosets (all from NEPRC). Of these, 8 marmosets were healthy, 11 marmosets had CLE, and 18 marmosets died or were euthanized because of other diseases, including intestinal adenocarcinoma ($n = 2$), chronic nephritis or renal disease ($n = 15$), hepatic lipidosis ($n = 4$), pancreatitis ($n = 2$), peritonitis ($n = 1$), cholecystitis ($n = 4$), steatohepatitis ($n = 1$), chronic prostatitis ($n = 1$), enteropathogenic *E. coli* ($n = 1$), and lymphoma and ulcerative enteritis ($n = 1$). The median serum cobalamin concentrations were 226 pg/mL (range, 213 to 627 pg/mL) in healthy marmosets, 225 pg/mL (149 to 514 pg/mL) in marmosets with CLE, and 297 pg/mL (149 to 663 pg/mL) in marmosets with other diseases; serum cobalamin concentration did not significantly differ among the 3 groups ($P = 0.148$). The median serum folate concentration was 141 ng/mL (range, 84 to 230 ng/mL) in

Table 7. Parallelism of serum folate concentration (ng/mL) of analyzer-diluted samples from common marmosets

Dilution	Observed folate concentration	CV (%)
Serum 1		8.7
1:10	not available	
1:20	not available	
1:40	629	
1:100	556	
Serum 2		20.6
1:10	209	
1:20	179	
1:40	160	
1:100	126	
Serum 3		11.9
1:10	not available	
1:20	not available	
1:40	488	
1:100	412	
Serum 4		9.2
1:10	not available	
1:20	322	
1:40	316	
1:100	271	
Serum 5		16.9
1:10	not available	
1:20	not available	
1:40	675	
1:100	690	

healthy marmosets, 68.6 ng/mL (18.3 to 226 ng/mL) in marmosets with CLE, and 203 ng/mL (28.3 to 327 ng/mL) in marmosets with other diseases; serum folate concentration differed significantly ($P = 0.001$) among the 3 groups. In particular, serum folate concentrations were significantly ($P = 0.008$) different between marmosets with CLE and those with other diseases.

The sensitivity of low serum cobalamin concentration (cutoff value, 322.0 pg/mL) for the diagnosis CLE in common marmosets was 44.4% (95% CI, 21.5% to 69.2%); and specificity was 81.8% (95% CI, 48.2% to 97.7%). The sensitivity and specificity of low serum folate concentration (cutoff value, 54.8 ng/mL) for the diagnosis of CLE were 71.4% (95% CI, 29.0% to 96.3%) and 73.9% (95% CI, 51.69% to 89.8%), respectively.

Given that several other diseases in the subject population, such as *E. coli* infection and intestinal carcinoma, involved the gastrointestinal tract, we classified the animals as healthy ($n = 8$), those with diseases of the gastrointestinal tract ($n = 15$), and those with diseases outside of the gastrointestinal tract ($n = 15$). Whereas serum cobalamin concentrations were not significantly different among the 3 groups ($P = 0.484$), serum folate concentrations were ($P = 0.040$), serum folate concentrations were significantly ($P = 0.036$) different between marmosets with gastrointestinal disease and those with extragastrointestinal diseases.

The sensitivity and specificity of a low serum cobalamin concentration (cutoff value, 322.0 pg/mL) for the diagnosis of gastrointestinal disease in common marmosets were 57.9% (95% CI, 33.5% to 79.8%) and 63.6% (95% CI, 30.8% to 89.1%), respectively. The sensitivity and specificity for a low serum folate concentration (cutoff, 54.8 ng/mL) for the diagnosis of gastrointestinal disease were 85.7% (95% CI: 42.1% to 99.6%) and 60.9% (95% CI: 35.5% to 80.3%), respectively.

Table 8. Recovery results after 1:1 spiking of marmoset serum samples with those of known serum folate concentration

	Expected concentration (ng/mL)	Observed concentration (ng/mL)	Observed/expected (%)
Serum 1: serum 2	11.4	11.1	97.7
Serum 1: serum 3	13.8	12.4	90.1
Serum 1: serum 4	15.1	15.6	103.2

Serum folate concentrations were: serum 1, 9.6 ng/mL; serum 2, 13.1 ng/mL; serum 3, 17.9 ng/mL; and serum 4, 20.6 ng/mL.

Table 9. Recovery results after spiking marmoset serum samples with manufacturer-provided folate standards

	Folate concentration (ng/mL)			Mean observed/ expected (%)
	Spiking solution	Observed	Expected	
Serum 1				82.2
	0 (diluent)	6.3	not applicable	not applicable
	3.0	7.7	7.8	98.2
	14.0	9.4	13.3	70.7
	21.8	13.4	17.2	77.8
Serum 2				86.1
	0 (diluent)	1.2	not applicable	not applicable
	3.0	2.9	2.7	109.6
	14.0	6.0	8.2	72.9
	21.8	9.1	12.1	75.7
Serum 3				81.8
	0 (diluent)	5.4	5.4	
	3.0	6.5	7.0	93.2
	14.0	9.7	12.5	77.6
	21.8	12.2	16.3	74.7
Serum 4				75.0
	0 (diluent)	3.2	not applicable	not applicable
	3.0	3.9	4.7	82.6
	14.0	7.0	10.2	67.9
	21.8	10.5	14.1	74.3
Serum 5				101.4
	0 (diluent)	4.2	not applicable	not applicable
	3.0	3.8	5.7	66.9
	14.0	14.5	11.2	129.3
	21.8	16.3	15.1	107.9

100 µL of the spiking sample was added to 100 µL of serum

In a cross-sectional study, serum cobalamin and folate concentrations were measured in 43 marmosets (all from NEPRC). According to a cutoff value of 322.0 pg/mL for low cobalamin, 15 of the 43 animals had a decreased serum cobalamin concentration. When 54.8 ng/mL was used as the cut-off value, 2 of the 43 animals had a decreased serum folate concentration.

In a longitudinal study, serum cobalamin and folate concentrations were measured in 4 animals (all from NEPRC) over several years (Figures 1 and 2). The final necropsy diagnosis and cobalamin and folate concentrations were available for review also (Table 11).

Discussion

Analytical validation is necessary when immunoassays are used for the first time in any species. In addition, species-specific reference intervals need to be generated, and often samples need to be diluted to bring them into the working range of an assay.⁴² We performed a partial analytical validation of commercially available assays for the measurement of serum cobalamin and folate concentrations in humans for use in common marmosets. The majority of the validation study was performed by

using pooled serum samples due to insufficient volume of samples from individual marmosets. This accommodation makes the detection of a possible matrix effect difficult. However, we showed that the automated cobalamin and folate assays we tested yielded linear, accurate, precise, and reproducible information from samples of marmoset serum. In the assessment of linearity using manual 2-fold dilution, the samples were at the lower end of the working range of the assay, thus producing the increased observed:expected ratios. However, when samples with higher concentrations were diluted by using the analyzer's built-in dilution function, their CV% were within the accepted criterion of less than 30% for immunoassays¹⁶ and very close to the stringent criterion of less than 20%,³⁴ with only a single out-of-range value (20.6%).

The use of banked samples did not affect the concentration measurements in the current study. From our limited data set, serum cobalamin and folate concentrations appear to be relatively stable when marmoset serum samples are stored at -80 °C for as long as 7 y.

The reference interval for cobalamin established for common marmosets (322 to 2642 pg/mL) is comparable to those for pig-tailed macaques (961 to 2000 pg/mL) and rhesus macaques

Table 10. Precision and reproducibility ($n = 5$ replicates) of measurement of serum folate concentration in common marmosets by using an automated analyzer

	Mean $\pm$ 1 SD (ng/mL)	CV (%)
Intraassay variability		
Serum 1	13.8 $\pm$ 0.5	3.7
Serum 2	18.2 $\pm$ 1.7	9.4
Serum 3	9.7 $\pm$ 0.5	5.4
Serum 4	22.2 $\pm$ 0.4	1.8
Interassay variability		
Serum 5	13.9 $\pm$ 0.8	6.0
Serum 6	16.8 $\pm$ 0.9	5.5
Serum 7	9.6 $\pm$ 0.4	3.7
Serum 8	21.8 $\pm$ 0.9	4.1

Figure 1. Serum cobalamin concentrations in 4 marmosets over several years.

(727 to 2000 pg/mL) generated by using the same assay.²² In addition, by using 2 different radioimmunoassays, plasma vitamin B12 concentrations in vervet monkeys (*Cercopithecus pygerythrus*) produced similar intervals: 1545 $\pm$ 451 and 1396 $\pm$ 450 pg/mL.⁴⁶

Serum folate concentrations in rhesus macaques from a microbiologic assay using *L. casei*³² were 15.0 $\pm$ 2.4 ng/mL and in the plasma of vervet monkeys by using 2 immunoassays were 10.2 $\pm$ 3.7 and 8.2 $\pm$ 2.2 ng/mL. These values are very different from the reference interval that we established for common marmosets in the current study (54.8 to 786.4 ng/mL) and probably reflects the methodology used.

Although serum cobalamin concentrations in marmosets did not differ significantly between the 2 primate centers evaluated (SNPRC and BMAC), serum folate concentrations did, and SNPRC marmosets had higher serum folate concentrations than those at BMAC. This difference can be attributed to 3 possible reasons: a dietary component, a diet treatment component, and differences in the gut microbiota. BMAC marmosets were fed a purified diet (TD.07148 Marmoset Diet, Harlan Teklad, Madison, WI) and a primate enrichment mixture consisting of nuts, seeds, and dried fruit from the same manufacturer. However, both of these diet components are irradiated at the manufacturing plant. SNPRC marmosets are on 2 diets, a commercial New World primate diet (AVP Callitrichid 5LK6, Mazuri, St Louis, MO) and the same purified diet (without irradiation) used at BMAC. In addition, marmosets at the SNPRC included daily food enrichment, including yogurt, fruits, and oat cereal, which are good supplemental sources of folate.¹⁵ Dietary supplementation of folate leads to increased plasma concentrations in other primate species.⁴⁶ Irradiation has been reported to decrease folic acid concentrations by 20% to 30% in various foods;⁴⁶ whether

Figure 2. Serum folate concentrations in 4 marmosets over several years.

irradiation had a similar effect in the current study is unknown. Folate is synthesized mainly by jejunal bacteria,²⁷ and differences in the fecal microbiome between the marmosets of these 2 colonies have been reported³⁶ and could be another reason for the differences in serum folate concentrations between the 2 centers. Given these observations, marmoset colony-specific reference intervals for serum folate concentrations may be appropriate for monitoring colony gastrointestinal health.

Although folate concentrations did not significantly differ between marmosets with a history of gastrointestinal disease and those without such a history, serum cobalamin concentrations did. However, only 3 of the 14 (21.4%) marmosets had serum cobalamin concentrations lower than the established reference interval. In a recent study,²² 5 of the 6 pigtail macaques with chronic diarrhea had decreased serum cobalamin concentrations, but all 6 rhesus macaques with chronic diarrhea had normal cobalamin concentrations. Cobalamin concentrations are low in some humans with inflammatory bowel disease⁵ and dogs with chronic enteropathy.⁷ A dietary cause for a decreased serum cobalamin concentration is unlikely in these marmosets, which were fed a commercial diet.²² Rhesus macaques fed cobalamin-deficient diets were asymptomatic for 12 to 18 mo despite severely decreased serum cobalamin concentrations.²⁶ Therefore, the absence of clinical signs is not sufficient to assume normal serum cobalamin concentrations. Other reasons for low cobalamin concentrations include genetic defects, changes in gut microbiota,³⁹ hyperthyroidism,¹² exocrine pancreatic insufficiency,⁴⁰ lymphoma,⁴⁰ ageing,⁴⁷ and metabolic causes.⁴¹

The diagnostic utility of decreased serum concentrations of cobalamin or folate (or both) for the diagnosis of CLE or gastrointestinal disease in common marmosets was suboptimal: low serum folate concentrations were moderately sensitive (greater than 70%) for gastrointestinal disease or CLE, and low cobalamin concentrations were specific (greater than 80%) for CLE. These features limit their utility in the diagnosis of CLE or occult gastrointestinal disease. Nonetheless, these measurements may serve as screening tests, because no antemortem tests are currently available. However, this situation does raise questions regarding the value of supplementation of these vitamins in animals with CLE or gastrointestinal disease. False-positive and false-negative rates for detecting cobalamin deficiency by using commercial assays are as high as 50% in humans.⁴¹ This is because these assays measure intrinsic factor binding of cobalamin as well as both serum holohaptocorrin and serum holotranscobalamin and can mask true deficiency or falsely imply a deficient state. For this reason, in humans, a low cobalamin concentration triggers the measurement of a functional biomarker such as methylmalonic acid or homocysteine, which are considered confirmatory.^{9,17} In addition, studies in dogs with chronic gastrointestinal disease have shown that only a subset of dogs has decreased cobalamin concentrations, and only a subset of

Table 11. Necropsy findings and serum cobalamin and folate concentrations in 4 marmosets from a single center (longitudinal study)

Animal	Necropsy findings	No. of days ^a	Cobalamin (pg/mL)			Folate (ng/mL)		
			Median	Minimum	Maximum	Median	Minimum	Maximum
1	CLE, nephritis	1775	>1001	882	>1001	156	104	772
2	CLE, cholecystitis, and nephritis	3514	391	391	>1001	106	15.9	529
3	Pancreatitis, hepatic amyloidosis, and cholecystitis	2283	224	149	362	158.5	103	300
4	No significant findings	2654	<149	<149	213	250	33.1	786

CLE, chronic lymphocytic enteritis

See Figure 1 for display of serum cobalamin concentrations over time.

See Figure 2 for display of serum folate concentrations over time.

^aNumber of days between the first and last samples available for testing.

these dogs have increased serum methylmalonic acid concentrations, suggesting a deficiency of cobalamin at the cellular level.^{7,8} Therefore, additional studies are warranted in common marmosets, to evaluate cobalamin and folate deficiencies at the cellular level.

The decreased folate concentrations in marmosets with CLE and gastrointestinal disease are interesting because marmosets raised on a folate-restricted diet develop clinical signs that terminated fatally in 59 to 136 d.¹⁸ These clinical signs included anorexia, weight loss, diarrhea, alopecia, ulceration of the oral and intestinal mucosa, anemia, leukopenia, and granulocytopenia, some of which signs have been reported in marmosets with CLE.²⁴ Serum folate measurements, when compared with cobalamin concentrations, are sufficient for the diagnosis of folate deficiency, and RBC folate measurement is indicated only rarely.²⁰

The fact that serum concentrations of cobalamin and folate from the marmosets from the same colony measured 120 and 220 d apart did not change significantly would suggest that the concentrations are stable over relatively long periods of time. The data from the 4 animals from the NEPRC show that normal marmosets can have very low cobalamin concentrations for long periods of time without any clinical signs (marmoset 4), and marmosets with CLE can have normal serum cobalamin and folate concentrations (marmoset 1). It is noteworthy, that in one animal with CLE, cobalamin and folate concentrations (marmoset 2) did decrease in over 600 d. Whether there are subsets of marmosets with CLE based on their response to diets, antibiotics, or immunosuppressive (steroids) drugs, as has been described for other species, including dogs, is unknown.²³

The major limitation of the current study is that it was based on archived samples. In addition, blood volume was a limiting factor, and pooled samples were used for the validation, which was suboptimal because we were unable to fully assess the presence of any matrix effects. Furthermore, a relatively small number of animals was used to establish reference intervals. In an ideal scenario, serum samples from at least 120 marmosets would have been used to establish reference intervals, as recommended by the International Federation of Clinical Chemistry.¹¹

In the current study, we validated commercially available human assays for the measurement of serum cobalamin and folate for use in common marmosets. In addition, we established reference intervals for serum cobalamin and folate concentrations in this species. The difference in serum folate concentration between centers suggests that facility-specific reference intervals may be more appropriate for this analyte. Low serum cobalamin and folate concentrations were present in some common marmosets with gastrointestinal disease and chronic lymphocytic enteritis. However, additional studies are necessary to further elucidate the utility of these measurements in these animals.

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