รายงานวิจัยฉบับสมบูรณ์

โครงการการวัดค่าของสาร 3-โบรโมไทโรซิน ในอุจจาระของสุนัขที่ปกติและสุนัขที่ป่วยด้วยโรคท้องเสียเรื้อรัง โดยใช้เทคนิคแกสโครมาโตกราฟฟี่แมสสเปกโตเมตรี่

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ผู้วิจัย ผศ.สพ.ญ.ดร.พันพิชา สัตถาสาธุชนะ
สังกัด ภาควิชาเวชศาสตร์คลินิกสัตว์เลี้ยง คณะสัตวแพทยศาสตร์
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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและต้นสังกัด

Abstract (บทคัดย่อ)

Studies that have used serum 3-bromotyrosine (3-BrY) to investigate eosinophil activation in dogs have found elevated 3-BrY levels in clinical patients with chronic enteropathy (CE). To our knowledge, a method to measure 3-BrY concentrations in feces has not been reported. We developed and analytically validated an electron ionization gas chromatography–mass spectrometry method to measure fecal 3-BrY concentrations in dogs. The mean and maximum fecal 3-BrY concentrations in healthy dogs (n = 40) and dogs with CE (n = 40) over 3 consecutive days were compared. Analytical validation had a limit of blank and a limit of detection of 2.5 and 3.7 mmol/g of feces, respectively. The mean coefficients of variation for precision and reproducibility for 3-BrY were 11.2% (range: 7.5–14.2%) and 10.1% (4.8–15.2%), respectively. The ranges of observed-to-expected ratios for linearity and accuracy were 81.3–125% and 85.4–120%, respectively. The reference intervals for mean and maximum fecal 3-BrY concentrations in 40 healthy dogs were 3.7–23.0 and 3.7–37.8 mmol/g of feces. Mean and maximum fecal 3-BrY concentrations in dogs with CE were significantly higher than those of healthy dogs ($p < 0.001$). Further research is warranted to determine the clinical usefulness of fecal 3-BrY concentrations in dogs with CE.

Keywords: 3-bromotyrosine; chronic enteropathy; dogs; eosinophils; feces; validation studies

Project Code: MRG6080007

Project Title: Evaluation of 3-bromotyrosine concentrations in fecal samples from healthy dogs and dogs with inflammatory bowel disease using gas chromatography/mass spectrometry

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Introduction

Chronic enteropathy (CE) is a group of intestinal diseases that lead to gastrointestinal (GI) signs such as diarrhea, vomiting, and weight loss that last >3 wk.[1,5,12] The histologic findings of CE are varied and can be characterized by the predominance of various inflammatory cell types, including lymphocytes, plasma cells, neutrophils, or eosinophils.[5,7,20] In dogs, eosinophilic gastroenteritis that is unrelated to parasitic infestation is classified as a subtype of CE.[18]

Noninvasive markers to assess eosinophil activation in the canine GI tract are limited. Invasive procedures, including GI biopsies, are generally used to diagnose eosinophil infiltration in dogs with CE. To avoid the risks associated with the anesthesia required to perform an intestinal biopsy and to decrease the financial burden on dog owners, a noninvasive method to identify intestinal eosinophilic inflammation is needed. 3-bromotyrosine (3-BrY) is a stable byproduct of eosinophil peroxidase generated after eosinophil activation.[18,23] Serum 3-BrY is used as a marker of eosinophil activation in human patients, especially those with asthma.[13,21,22] We previously reported an assay to measure 3-BrY concentrations in dog serum using electron impact gas chromatography–mass spectrometry (EI-GC/MS).[16] Furthermore, other studies have reported elevated serum 3-BrY concentrations in dogs with CE.[15,17] Serum markers, unlike those in fecal samples, are not specific to the GI tract. Various other markers for GI diseases, such as canine alpha$_1$-proteinase inhibitor, calprotectin, N-methylhistamine, and S100A12, are measured in fecal samples to assess patients with CE.[4,9-11,14]

Our objectives were 1) to develop an assay to measure 3-BrY concentrations in canine fecal samples, 2) to analytically validate the assay, 3) to determine reference intervals for fecal 3-BrY concentrations in healthy dogs, and 4) to compare the fecal 3-BrY concentrations of healthy dogs with those of dogs with CE.

Materials and methods

All fecal samples of healthy dogs in the control group and dogs with CE procedures were collected after approval by the Texas A&M University Institutional Animal Care and Use Committee (IACUC 2012-101). For analytical validation, 10 excess fecal samples (1 g of feces each) from diagnostic submissions to the Gastrointestinal Laboratory at Texas A&M University (College Station, TX) were collected into pre-weighed polypropylene tubes and stored at –20°C up to 7 d until extraction. To establish a reference interval for fecal 3-BrY concentrations in the healthy dogs, fecal samples were collected for 3 consecutive days from 40 healthy control dogs (Table 1). All dogs had been regularly vaccinated and dewormed, did not have clinical signs of disease, and did not receive any medications. In addition, 40 dogs with CE were enrolled in the study (Table 1). CE was diagnosed if dogs had chronic GI signs that persisted >3 wk, and if secondary causes of these signs, including GI parasites, GI neoplasia, pancreatitis, exocrine pancreatic insufficiency, renal failure, and hepatic failure could be eliminated.[1,5] Fecal samples from dogs with CE were obtained over 3 consecutive days in the same manner as samples from healthy control dogs. Fecal samples were stored at –20°C up to 7 d until extraction.

Fecal samples were thawed at room temperature and diluted 1:5 in a fecal extraction buffer composed of phosphate-buffered saline (BupH; Thermo Fisher Scientific, Rockford, IL) with 5% newborn calf serum (Sigma-Aldrich, St. Louis, MO), 1% Triton X-100 (Surfact-Amps X-100; Thermo Fisher Scientific), and 0.25 mM thimerosal (Sigma-Aldrich). After homogenization by vigorous shaking for 20 min at room temperature, suspensions were centrifuged at 5°C for 20 min at 2,100 × *g*. Supernatants were collected using serum filters (Fisherbrand IB model; Thermo Fisher Scientific) and centrifuged at room temperature for 30 min at 10,600 × *g*. The final supernatants (fecal extracts) were stored frozen at –80°C up to 6 mo until analysis.

D_3-bromotyrosine (D_3-BrY) was prepared as described previously.[16,17] The reaction was started by combining D_4-L-tyrosine (L-tyrosine [RING-D_4, 98%]; Cambridge Isotope Laboratories, Tewksbury, MA) with N-bromosuccinimide (Sigma-Aldrich) in water for 1 h at 37°C. The D_3-BrY was purified and quantified by reverse-phase high-performance liquid chromatography (HPLC) using a C18 column (Gemini NX50, 4.6 × 250 mm; Phenomenex, Torrance, CA) with a solvent mixture of 65% of 0.1% trifluoroacetic acid (TFA; Sigma-Aldrich) in water (HPLC-grade water; Burdick & Jackson, Morristown, NJ), pH 2.5, and 35% of 0.1% TFA in methanol (Sigma-Aldrich), pH 2.5. The D_3-BrY was stored at –80°C up to 6 mo until use.

Eight nmol of D_3-BrY was added to the mixture of 250 µL of extracted fecal samples, 250 µL of water, and 1.5 mL of 0.1% TFA, pH 5.0. The mixture was centrifuged at 4°C for 15 min at 16,000 × *g*. The supernatants were applied to solid-phase extraction columns (Supelclean ENVI-18; Sigma-Aldrich) to remove polar compounds. 3-BrY was eluted with 1.6 mL of 25% methanol from the solid-phase extraction column. The eluent was filtered using a polyethersulfone membrane (Captiva premium syringe filter with 0.45-µm polyethersulfone membrane; Agilent Technologies, Santa Clara, CA). The sample was completely dried in a rotary vacuum device at 45°C for 6 h. For derivatization, 100 µL of acetonitrile (Thermo Fisher Scientific), 40 µL of diisopropylethylamine (Sigma-Aldrich), and 40 µL of ethyl heptafluorobutyrate (Sigma-Aldrich) were added to the dried sample at 4°C. After sonicating for 1 h, the reaction mixture was concentrated under a nitrogen stream at room temperature, and 30 µL of N-methyl-N-(t-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA; Thermo Fisher Scientific) was added to the mixture. The sample was completely dried under a nitrogen stream and reconstituted in 70 µL of 25% MTBSTFA in undecane (Sigma-Aldrich). The sample was centrifuged at 16,000 × *g* for 15 min. One µL of supernatant was used to measure the 3-BrY concentration. An EI-GC mass spectrometer

(Agilent Technologies) was equipped with capillary columns (VF-17ms, 30 ms, 0.25 × 0.25 µm; Agilent Technologies) using helium gas as the mobile phase. The injector, transfer line, and source temperatures were set at 180°C, 300°C, and 250°C, respectively. The initial oven temperature was maintained at 180°C for 1 min and then increased at a rate of 40°C/min and held at 310°C for 5 min. Determination of 3-BrY concentration was based on internal standard calibration using the D_3-BrY isotope. The fragment ions at *m/z* of 257 and 260 were monitored for 3-BrY and D_3-BrY, respectively. Commercially available 3-BrY (3-bromo-L-tyrosine; BOC Science, Shirley, NY) was used as a reference standard for the validation of analytical methods. The 3-BrY standard working range (0, 0.5, 1, 2.5, 5, 10, 20, 30, 40, and 50 µmol/L) was established using previously published protocols.[16]

The limit of blank (LOB), limit of detection (LOD), precision, reproducibility, linearity, and accuracy were determined.[2,3] LOB was calculated by measuring 3-BrY concentrations in 6 blank samples, using the equation: $\text{mean}_{\text{blank}} + 2(\text{SD}_{\text{blank}})$. LOD was calculated using the lowest fecal 3-BrY concentration that the assay could detect, using the equation: $\text{LOB} + 2(\text{SD}_{\text{low concentration sample}})$. Precision was calculated by determining the intra-assay coefficient of variation (CV%) for 5 different fecal samples measured 6 times within the same assay run. Reproducibility was calculated using the inter-assay CV% for 5 different fecal samples, each analyzed in 6 consecutive assays on 6 different runs. Linearity was determined by calculating observed-to-expected (O/E) ratios for 5 different fecal samples serially diluted 1/2, 1/4, 1/8, and 1/16. Accuracy was evaluated by calculating O/E ratios for 5 different fecal samples that were spiked with 4 different 3-BrY concentrations (2.5, 5, 10, and 20 µmol/L).

Commercially available statistical software packages (JMP Pro 10, SAS Institute, Cary, NC; PRISM v.6.0, GraphPad Software, La Jolla, CA) were used for statistical analyses. A Shapiro–Wilk *W* test was used to assess the normality of the data. The categorical variables

comparison, including sex and breed size, was performed using the Pearson chi-squared test. A Student *t*-test was performed to compare ages. The reference intervals for mean and maximum fecal 3-BrY concentrations in healthy dogs were calculated using Microsoft Excel Freeware Reference Value Advisor.[6] A Mann–Whitney U test was used to compare fecal 3-BrY concentrations of the mean and maximum fecal 3-BrY concentration from 3 consecutive days of the healthy control dogs with those of the dogs with CE. The statistical significance was set at $p \leq 0.05$.

Results

LOB and LOD for the measurement of 3-BrY in fecal samples were 2.5 and 3.7 mmol/g of feces, respectively. Intra-assay CV%s were 7.5–14.2%, and inter-assay CV%s were 4.8–15.2% (Table 2). The O/E ratios for serial dilutions were 81.3–125% (Table 3). The O/E ratios of accuracy were 85.4–120% (Table 4).

The demographic characteristics of the dogs, including age ($p = 0.174$), sex ($p = 0.499$), and breed size ($p = 0.073$), were not significantly different between the 2 groups (Table 1). The median (range) of the mean and maximum fecal 3-BrY concentrations for the healthy control dogs was 5.4 (≤3.7–23.0) and 7.8 (≤3.7–38.1) mmol/g, respectively. The reference intervals for the 3-day mean and maximum fecal 3-BrY concentrations for healthy dogs, determined using nonparametric methods, were 3.7–23.0 and 3.7–37.8 mmol/g of feces, respectively (Fig. 1). The 3-day mean fecal 3-BrY concentration for the dogs with CE (median [range]: 33.7 [≤3.7–142] mmol/g) was significantly higher than that for the healthy control dogs ($p < 0.001$). The 3-day maximum fecal 3-BrY concentration for the dogs with CE (52.7 [≤3.7–198] mmol/g) was significantly higher than that for the healthy control dogs ($p < 0.001$).

Discussion

We established successfully a method for measuring 3-BrY concentrations in fecal samples using EI-GC/MS. Quantification of 3-BrY was based on calibration against an internal standard of the D_3-BrY isotope. Our analysis indicated that the most accurate molecular weights representing 3-BrY and D_3-BrY were 257 and 260, respectively.

This method for quantifying 3-BrY concentrations in canine fecal samples is more sophisticated than the previously reported method in serum. An additional extraction step was required before applying fecal samples to solid-phase extraction; however, this step was similar to one required for other fecal assays, such as an ELISA to measure fecal alpha$_1$-proteinase inhibitor[10] in dogs or a GC/MS-based assay to measure N-methylhistamine.[4] The fecal 3-BrY concentrations were calculated for the wet weight of the fecal samples and expressed in mg/g of feces.[9] The median (range) CV% of the 3-BrY concentrations for the 3 consecutive days of fecal samples in the healthy dogs and the dogs with CE was 21.8% (0–82.3%) and 47.7% (0–155%), respectively. Our results indicated a large variation in 3-BrY concentrations in the fecal samples from each dog; therefore, the 3-day mean and 3-day maximum of fecal 3-BrY concentrations were determined to account for this variability, as has been done for the fecal concentrations of other marker molecules.[13,14,16]

We found that the mean CV% for intra- and inter-assay variation for the fecal samples was 11.2% and 10.1%, respectively. Compared with our previous results, the mean CV% for fecal samples was higher than for serum samples.[16] However, the results of intra- and inter-assay variability for the measurement of fecal extracts was still within 15%, which is generally considered to be acceptable.[3,8] The O/E ratios for dilutional parallelism and spiking recovery should fall within 80–120%.[3,8] We found a $\bar{x}$ ± SD of linearity and accuracy of 101 ± 12% and 104 ± 8%, respectively. Because higher dilutions lead to fecal 3-BrY

concentrations near the LOD of the assay, our study suggested that the sample can be diluted up to 1:8 fold.

The activation of eosinophils potentially drives the eosinophil peroxidase–H_2O_2–bromine system.[13,19,23] 3-BrY is a specific byproduct of eosinophil peroxidase, which is released after the activation of eosinophils.[18,19] In our study, fecal 3-BrY concentrations in the healthy control dogs were relatively low, which could be explained by a lack of eosinophil activation in these dogs. This finding is similar to previously reported 3-BrY concentrations in the serum of healthy dogs.[16] We also found that the fecal 3-BrY concentrations in dogs with CE were higher than those in the healthy control dogs.

To our knowledge, a study has not been reported previously of a noninvasive method to measure a biomarker for eosinophilic gastroenteritis in dogs by determining 3-BrY concentrations in fecal samples. Measuring 3-BrY concentrations in fecal samples may prove to be a superior biomarker for eosinophil activation in the GI tract than serum biomarkers; the concentration of 3-BrY in fecal samples is more likely to reflect the activation of eosinophil infiltration in the GI tissue rather than other organ systems. Fecal 3-BrY concentrations in the dogs with CE evaluated in our study were greater than serum 3-BrY concentrations in the dogs evaluated in a previous study.[16] However, further cohort study should be performed to investigate the correlation between paired fecal and serum samples.

CE can be subclassified into 3 categories: food-responsive diarrhea, antibiotic-responsive diarrhea, and steroid-responsive diarrhea.[1,15] However, dogs with CE in our study were not classified into these 3 subtypes, which is a limitation of our study. Nonetheless, the development of a less invasive modality to diagnose CE may still prove to be beneficial for early diagnosis and treatment without the need to perform an intestinal biopsy. The relationship between eosinophilic infiltration in the GI tract and fecal 3-BrY concentrations should be further investigated.

An additional limitation of our study includes the lack of a standardized method to rule out the presence of GI parasites. Although the dogs' primary-care veterinarians indicated that the dogs did not have GI parasites prior to enrollment as determined by performing a fecal floatation and/or a fenbendazole treatment trial, we cannot exclude this possibility. In addition, the stability of fecal 3-BrY concentrations has not been fully determined. Nonetheless, the stability of serum 3-BrY stored at 4°C, –20°C, and –80°C has been shown previously to be stable for up to 7 d, 30 d, and 180 d, respectively.[17] The stability of fecal 3-BrY concentrations in different storage conditions should be further investigated.

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Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Table 1. Demographic distribution of 40 healthy control dogs and 40 dogs with CE for comparison of fecal 3-bromotyrosine concentrations.

Characteristic	Healthy dogs	Dogs with CE	*p* value
Age* (y)	4.5 (1–15)	7.0 (<1–13)	0.174
Sex† (*n*)			0.499
Male	21 (52)	24 (60)	
Female	19 (48)	16 (40)	
Breed size† (*n*)			0.072
Small (<10 kg)	17 (42)	9 (22)	
Medium (10–20 kg)	8 (20)	6 (15)	
Large (>20 kg)	15 (38)	25 (62)	

CE = chronic enteropathy.

* Mean, with range in parentheses.

† Numbers in parentheses are percentages.

Table 2. Precision (intra-assay variability) and reproducibility (inter-assay variability) of 3-bromotyrosine fecal extracts in fecal samples from 5 healthy dogs.

Sample	Repeats	Mean (mmol/g of feces)	SD	CV (%)
Intra-assay variability				
I	6	18.5	0.5	13.3
II	6	21.0	0.6	14.2
III	6	49.4	1.2	12.0
IV	6	66.4	1.0	7.5
V	6	112.5	2.0	9.1
Inter-assay variability				
I	6	27.3	0.8	15.2
II	6	29.5	0.8	13.2
III	6	35.7	0.3	4.8
IV	6	49.8	1.2	12.0
V	6	116.1	1.3	5.4

CV = coefficient of variation; SD = standard deviation.

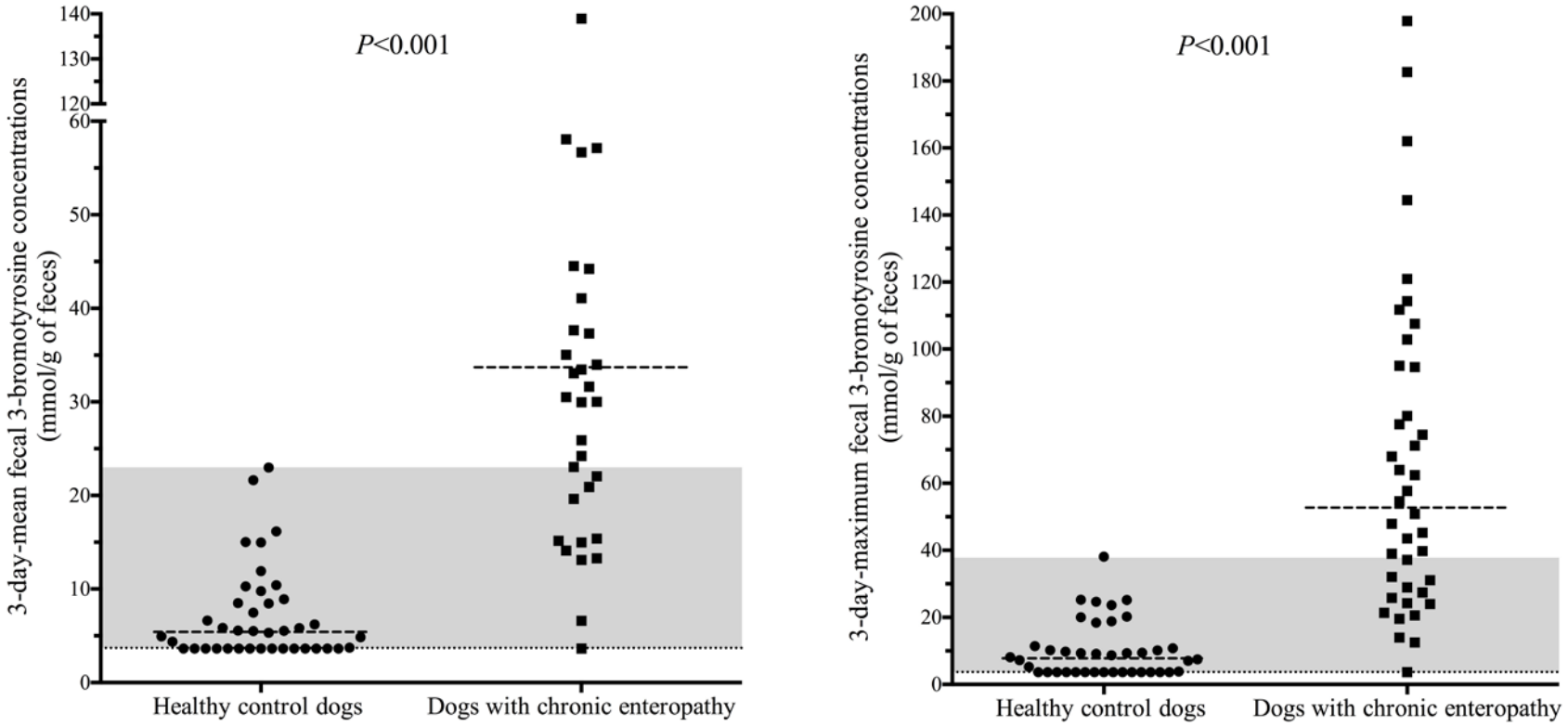
Table 3. Linearity (dilutional parallelism) of 3-bromotyrosine in fecal samples from 5 healthy dogs.

Sample/Dilution	Observed concentration (mmol/g of feces)	Expected concentration (mmol/g of feces)	Observed-to-expected ratio (%)
I			
Undiluted	38.6		
1:2	19.0	19.3	98
1:4	8.9	9.7	92
1:8	4.7	4.8	97
1:16	2.4	2.4	100
II			
Undiluted	48.1		
1:2	24.7	24.1	103
1:4	12.3	12.0	102
1:8	5.7	6.0	95
1:16	2.6	3.0	85
III			
Undiluted	51.8		
1:2	30.7	25.9	118
1:4	14.4	13.0	111
1:8	5.9	6.5	90
1:16	2.8	3.3	85
IV			
Undiluted	63.8		
1:2	34.0	31.9	106
1:4	19.0	16.0	119
1:8	7.2	8.0	90
1:16	3.3	4.0	81
V			
Undiluted	126.0		
1:2	68.5	63.0	109
1:4	31.6	31.5	100
1:8	17.7	15.8	112
1:16	9.8	7.9	125

Table 4. Accuracy (spiking recovery) of 3-bromotyrosine in fecal samples from 5 healthy dogs.

Sample	Spike concentration (µmol/L)	Observed concentration (mmol/g of feces)	Expected concentration (mmol/g of feces)	Observed-to-expected ratio (%)
I		23		
	2.5	38	35	106
	5.0	49	48	103
	10.0	87	73	120
	20.0	135	123	110
II		35		
	2.5	47	48	98
	5.0	63	60	105
	10.0	99	85	116
	20.0	136	135	101
III		62		
	2.5	75	75	101
	5.0	102	90	114
	10.0	129	112	115
	20.0	168	162	104
IV		90		
	2.5	97	103	94
	5.0	114	116	99
	10.0	160	140	114
	20.0	223	190	117
V		98		
	2.5	107	111	97
	5.0	112	123	91
	10.0	126	148	85
	20.0	170	198	86

Figure 1. Scatter plots of 3-bromotyrosine (3-BrY) concentrations from fecal extracts in 40 healthy control dogs and 40 dogs with chronic enteropathy. Each dot represents the **A.** 3-day mean or **B.** 3-day maximum fecal 3-BrY concentration of 1 dog. Medians are shown as dashed horizontal lines. The reference intervals of the 3-day mean and 3-day maximum fecal 3-BrY concentrations are shaded in gray.



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Dear Dr. Sattasathuchana:

I am pleased to inform you that your revised manuscript 18-0221.R3 entitled "Analytical validation of fecal 3-bromotyrosine concentrations in healthy dogs and dogs with chronic enteropathy" has been accepted for publication in the Journal of Veterinary Diagnostic Investigation. Accordingly, you may now cite the article as "in press" in J Vet Diagn Invest.

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Sincerely, on behalf of Grant Maxie, Editor-in-Chief,

Holly M. Farrell
Managing Editor
Journal of Veterinary Diagnostic Investigation
editorial@aavld.org

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