REFERENCE INTERVALS

Establishment of an RI for the urine ammonia-to-creatinine ratio in dogs

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Abstract

Background: Ammonia is produced and excreted by the kidney, contributing to systemic acid-base homeostasis through the production of bicarbonate. Disorders of acid-base balance can lead to many clinical problems and measuring ammonia excretion helps in determining if the kidneys are responding to acid-base challenges appropriately. Reference intervals are integral to clinical decision-making, and there is no current RI for the urine ammonia-to-creatinine ratio (UACR) in dogs.

Objective: This study aimed to generate an RI for the UACR in healthy adult dogs.

Methods: The study used adult, client-owned dogs that were presented to the University of Florida Primary Care and Dentistry service (n = 60). Physical examinations were performed and serum chemistry and urinalysis samples were obtained. Urine ammonia and creatinine concentrations were determined. Dogs were excluded if there were significant abnormalities in either their urinalysis or serum chemistry results. The RI for the UACR was calculated according to the recommendation of the American Society for Veterinary Clinical Pathology. Data were evaluated for correlation with serum bicarbonate, weight, age, and sex.

Results: The RIs for the UACR were 0.16-23.69 with 90% confidence intervals for the lower and upper limits of (0.13-1.17) and (20.50-23.75), respectively. No significant impact of age, sex, or weight was found. There was no discernable relationship between serum bicarbonate and UACR.

Conclusions: Establishing an RI for UACR in healthy adult dogs will allow for further studies to determine if alterations are observed during specific disease states.

KEYWORDS
acid-base, assay, canine, kidney, renal physiology, solute transport

1 | INTRODUCTION

Maintaining acid-base homeostasis is critical for health. The kidneys have two major functions in acid-base homeostasis: reabsorbing filtered bicarbonate and generating new bicarbonate. Complete reabsorption of filtered bicarbonate, while critical, is not enough to maintain acid-base homeostasis. The kidneys must generate new bicarbonate to replenish the bicarbonate consumed in the buffering of endogenous acids, which involves both ammonia metabolism and titratable acid excretion. Ammonia metabolism leading to ammonia

excretion is the greater component of new bicarbonate generation under basal conditions and in response to acidosis.[1,2] Ammonia is generated from amino acid metabolism, predominantly glutamine, and the complete metabolism of glutamine in the proximal tubule leads to the equimolar generation of NH_4^+ and HCO_3^-.[2] Thus, ammonia excretion is the primary source of new bicarbonate generation in the kidneys.

Disorders of acid-base homeostasis result in clinical problems such as electrolyte disturbances, increased susceptibility to cardiac arrhythmias, bone disorders, and skeletal muscle atrophy.[3] A significant proportion of human patients with chronic kidney disease (CKD) develop metabolic acidosis, including as many as 40% of those with severe CKD, and inadequate ammonia excretion appears to be the driving factor.[4,5] Moreover, impaired ammonia excretion in human patients with CKD correlates with worse clinical outcomes, including progressive CKD and death.[6,7]

Metabolic acidosis is also recognized as a clinical problem in canine CKD patients.[8] In a research laboratory setting, the canine kidney has been shown to use glutamine to generate ammonia and bicarbonate, which increases in response to an exogenous acid load, similar to the human kidney.[9] However, canine ammonia excretion has not been evaluated in either healthy dogs or during disease states such as CKD.

Thus, our primary objective was to generate an RI for the canine urinary ammonia-to-creatinine ratio (UACR) from a population of adult dogs presenting to the primary care service of a veterinary teaching hospital. We also evaluated possible correlations between UACRs and sex, body size, and serum bicarbonate concentrations.

2 | MATERIALS AND METHODS

2.1 | Study population

Apparently healthy dogs aged >1 year, of any sex or neuter status, were included. Dogs were client-owned patients of the Primary Care and Dentistry Service at the University of Florida Small Animal Hospital. Health status was assessed based on history, physical examination findings, serum chemistry, and urinalysis. Dogs were excluded if there was azotemia, an active urine sediment, or evidence of significant systemic disease that would alter renal function. Azotemia was defined as blood urea nitrogen concentration >36 mg/dL or creatinine concentration >1.4 mg/dL. Dogs were excluded if urine samples exhibited gross hematuria and if there was cytologic evidence of inflammation (≥5 WBC/high power field), or bacteriuria. Mild cases of dental disease, osteoarthritis, or dermatitis, and heart murmurs without evidence of clinical cardiac dysfunction, were not considered reasons for exclusion. Dogs were also excluded if they were receiving glucocorticoids, diuretics, or angiotensin-converting-enzyme inhibitors. Other chronic medications were permitted if these medications had no known impact on creatinine or ammonia metabolism. This study was approved by the University of Florida Institutional Animal Care and Use Committee

and Veterinary Hospitals Research Review Committee, and written consent was obtained from the owners.

2.2 | Sample collection

Sampling occurred at the hospital. At least 5 mL of urine was obtained from each dog by cystocentesis (n = 5) or midstream collection of a voided urine sample (n = 43) into a clean container. The method of urine collection was left to the discretion of the primary clinician overseeing the case. After collection, 0.5-1 mL of urine was placed in a sterile conical tube with 1 mL of mineral oil to prevent evaporation and stored at room temperature for less than 4 hours. The mineral oil was used to help prevent evaporation because it has negligible matrix effects of <1% in measured values for creatinine and ammonia (Data S1, Tables 1 and 2). Samples were centrifuged at 2100g for 2 minutes (Thermo Scientific Sorvall Legend micro21R) with 400 μL of supernatant placed into microcentrifuge tubes and stored at −80°C until the time of assay. Although no stability data exist for ammonia in canine urine, previous work with human urine found that urinary ammonia remains stable when stored at −22°C

TABLE 1 Descriptive statistics and nonparametric reference intervals for canine urine ammonia: creatinine ratio (UACR) with and without outliers

Category	All individuals (n = 48)	Outliers removed (n = 41)
UACR mean (SD)	21.6 (38.59)[a]	8.29 (6.13)[a]
UACR median	9.11[a]	7.09[a]
Q1-Q3	3.78-15.47[a]	3.10-11.49[a]
Range	0.13-183.63[a]	0.13-23.75[a]
Reference interval	0.26-172.07	0.16-23.69
90% CI for lower limit	0.13-1.39	0.13-1.17
90% CI for upper limit	111.49-183.63	20.50-23.75

Abbreviations: CI, confidence interval; mean, median, interquartile ranges (Q1-Q3), and ranges reflect untransformed values; SD, standard deviation; UACR, urine ammonia-to-creatinine ratio.
[a]Data were not normally distributed.

TABLE 2 Generalized linear model of the canine urine ammonia: creatinine ratio (UACR) and bicarbonate, weight, age, and sex

Coefficients	Estimate	Standard error	t-value	P-value
Intercept	12.81	11.91	1.08	.29
Bicarbonate	−0.09	0.46	−0.19	.85
Weight	−0.08	0.11	−0.77	.45
Age	−0.08	0.35	−0.23	.82
Sex	0.63	2.13	0.30	.77

Note: Multiple R^2: .02, adjusted R^2: −.09; F-statistic 0.16 on 4 and 26 *df*, P-value .96. $P < .05$ was considered significant.

for up to 12 months.[10] Our samples were stored between 39 and 244 days prior to sample processing.

2.3 | Analytical methods

Urine ammonia concentrations were measured using a commercially available enzymatic assay (Ammonia Reagent Assay; Pointe Scientific). Urine creatinine concentrations were measured using the modified Jaffe method with a commercially available assay (Creatinine Assay Kit [ab204537]; Abcam). The CV and total observed error (TEobs) for both assays were calculated based on the American Society for Veterinary Clinical Pathology (ASVCP) guidelines for allowable total error (TEa).[11] The CV and TEobs were calculated as follows:

$$CV\% = \left(\frac{\text{Standard deviation}}{\text{Mean}} \right) \times 100,$$

$$TEobs\% = 2CV + bias(\%).$$

The intra- and inter-assay CVs were calculated using quality control materials provided by the assay companies. The intra-assay CV was 1.27%, the inter-assay CV was 2.34%, and the TEa was 6.0%. For creatinine, the intra-assay CV was 1.27%, the inter-assay CV was 2.54%, and the TEa was 3.5%. A SpectrMax ABS plus microplate reader (Molecular Devices) was used for measurements. To keep units consistent for ammonia and creatinine, measurements for the latter were converted from mg/dL to mmol/L by multiplying by 0.055. The ammonia-to-creatinine ratio was then calculated using the following formula:

$$UACR = \frac{\text{Urine ammonia concentration}}{\text{Urine creatinine concentration}}.$$

The University of Florida Small Animal Clinical Pathology Laboratory performed all urinalyses as part of the inclusion criteria. This included a urine specific gravity evaluation using a refractometer and semi-quantitative dipstick analysis, including a pH measurement.

2.4 | Statistical analysis

The number of dogs enrolled in this study was informed by the ASVCP guidelines.[12] Statistical analysis was completed with the R statistical program (https://www.r-project.org/) and Microsoft Excel 2013 for Windows (Microsoft Corp.) with the Reference Value Advisor v2.1 add-in (freeware v2.1: http://www.biostat.envt.fr/reference-value-advisor). An RI for UACR in this population of dogs was determined nonparametrically and comprised the central 95% of the fitted distribution with 90% confidence intervals calculated around the lower (2.5%) and upper (97.5%) limits. The confidence intervals

were also assessed in relation to the width of the RIs, as was previously recommended.[12] The Dixon method was used to detect outliers. The Anderson-Darling test was used to assess for normality, and statistical significance was set at $P < .05$ for all analyses.

All factors assessed, including UACR, age, breed, sex, and weight, were summarized descriptively. A generalized linear model of UACR was fitted with serum bicarbonate, age, sex, and weight as cofactors.

3 | RESULTS

3.1 | Study population

Sixty dogs were enrolled in the study from August 2019 to October 2020. Twelve dogs were excluded based on defined study criteria, leaving 48 in the study population (Figure 1). There were 19 male neutered, two male intact, 25 female neutered, and two female intact dogs. This population included the following breeds; mixed breed (n = 16), pit bull (n = 5), Golden Retriever (n = 3), Great Dane (n = 3), Labrador Retriever (n = 3), Akita (n = 2), Bulldog (n = 2), Staffordshire Terrier (n = 2), Afghan (n = 1), Basset Hound (n = 1), Black Mouth Cur (n = 1), Carolina Dog (n = 1), Doberman (n = 1), German Shorthaired Pointer (n = 1), Greyhound (n = 1), Italian Greyhound (n = 1), Jack Russell Terrier (n = 1), Kerry Blue Terrier (n = 1), Miniature Dachshund (n = 1), and Springer Spaniel (n = 1). Ages ranged from 1.8 to 13.2 years (median 6.5 years). Weights ranged from 4.6 to 56 kg (median 27.2 kg).

3.2 | Reference interval

Data for urine ammonia concentrations were nonnormally distributed (Anderson-Darling, $P < .05$). Urine ammonia concentrations ranged from 2.1 to 1013.7 mmol/L (median 61.8 mmol/L). Urinary creatinine concentration values were normally distributed (Anderson-Darling, $P = .41$), with a range from 0.49 to 19.47 mmol/L (mean 8 mmol/L). Data for UACRs were clearly skewed (Figure 2); however, log transformation obtained an approximate Gaussian distribution (Anderson-Darling, $P > .05$). To establish an RI for UACRs, the lower 2.5% and upper 97.5% limits were determined using nonparametric methods. The RI for UACRs in dogs was 0.26-172.07 (Table 1). Seven dogs were identified as outliers by the Dixon method. The removal of these outliers had little effect on the median UACR, 9.11 and 7.09, respectively. With the exclusion of outliers, the RI for UACRs in dogs was 0.16-23.69 (Table 1). The 90% confidence interval around the lower limit was 0.13-1.17, and the 90% confidence interval around the upper limit was 20.50-23.75. The width of both confidence intervals compared with the width of the RIs was <0.2. The values for the lower and upper limits were 0.044 and 0.138, respectively. A generalized linear model did not detect significant relationships between UACRs and serum bicarbonate, sex, weight, or age (Table 2).
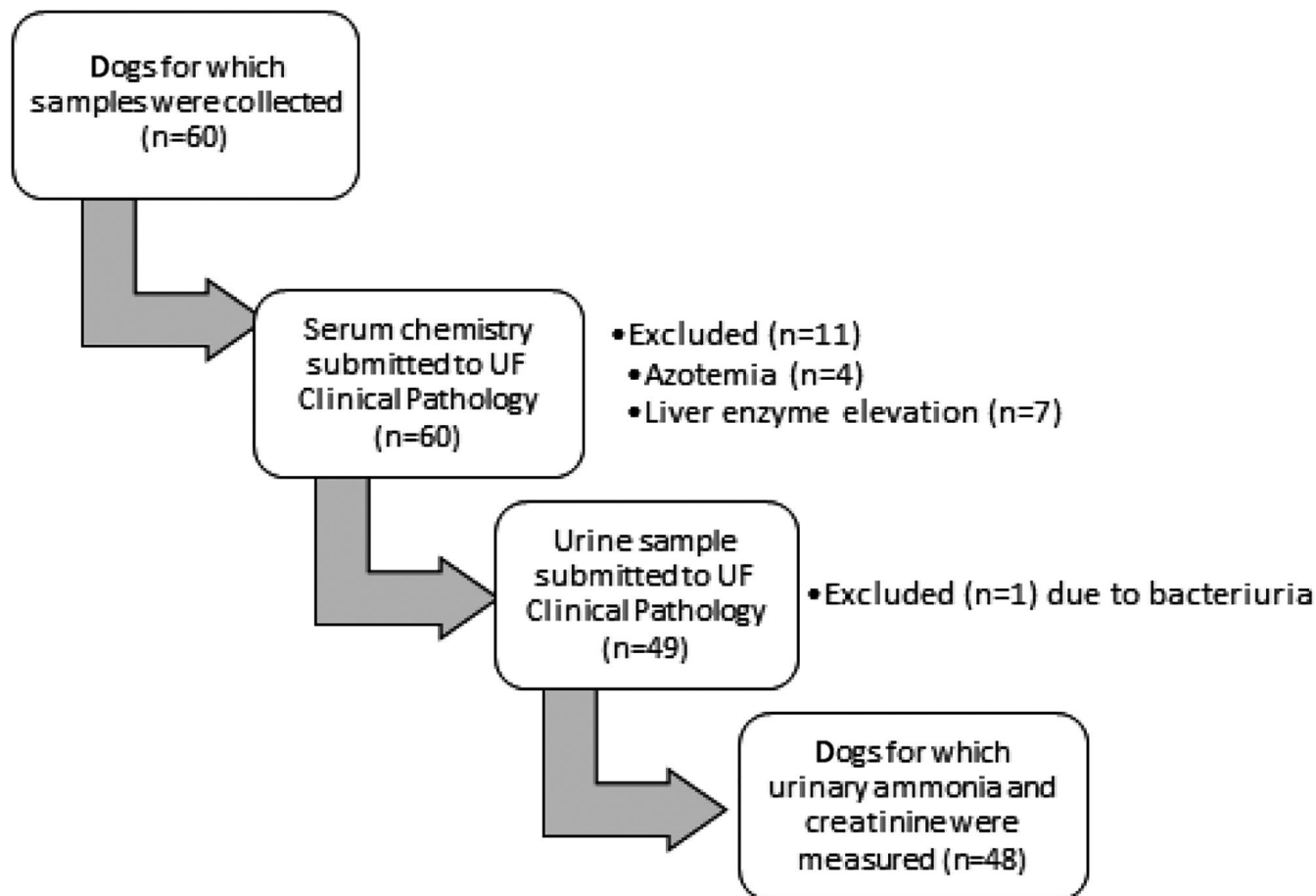
**FIGURE 1** Schematic of enrollment and exclusion criteria for dogs in this study. UF, University of Florida

4 | DISCUSSION

The primary objective of this study was to establish an RI for UAC excretion in healthy adult dogs. To achieve this, urine ammonia and creatinine concentrations were measured to calculate a UACR. In people, there are two proposed methods to estimate urinary ammonia excretion—the urinary anion gap and the urinary osmolal gap.[13] Using the urinary anion gap ($=U_{Na}^+ + U_K^+ - U_{Cl}^-$) to estimate ammonia excretion does not consider ammonia excretion with anions other than chloride.[14] The urinary osmolal gap ($=[2 \times (U_{Na}^+ + U_K^+) + U_{UN}/2.8 + U_{glucose}/18]$) represents the concentration of ammonia with its anion; however, it assumes the absence of active substances, such as unmeasured cations.[15] Previous studies comparing estimates of urinary ammonia excretion to direct measurements of urinary ammonia showed that these estimates are inaccurate.[16] The "gold standard" in human medicine is the direct measurement of urinary ammonia, which is then reported as either daily excretion (if 24-hour urine volume is known) or as the UACR.

We did not find evidence that the UACR was correlated with serum bicarbonate, sex, weight, or age. One possible explanation for the lack of sex differences found in this study might be the neutered status in the majority of our sample population (44/48).

There was no evidence that the four intact animals in the study were sequestered toward either end of the range of UACR values. However, given the small number of intact animals, conclusions should not be drawn based on these data. Future studies evaluating ammonia excretion in intact dogs would be needed to further evaluate for possible sex differences. The lack of correlation between UACs and serum bicarbonate concentrations is expected given the adequate renal function in this patient population. Similarly, the lack of a correlation between the UACR and weight is not surprising given the wide range of patient body condition scores and our inability to reliably account for differences in lean body mass.

A UACR RI was established for clinically healthy adult dogs in this study, but the sample size of 60 individuals should be considered when interpreting RIs. Previous work recommends that confidence intervals should not exceed 0.2 times the width of the RI.[12] When confidence intervals are within this limit, as is the case for our data, there is more evidence to support that the UACR RI established in this study is robust despite the smaller sample size. When outliers were excluded, the upper reference limit was 23.69, whereas when the outliers were included, the upper reference limit was 172.07. The dogs identified as outliers were examined for potential causes of increased UACRs. Two of the outliers had the
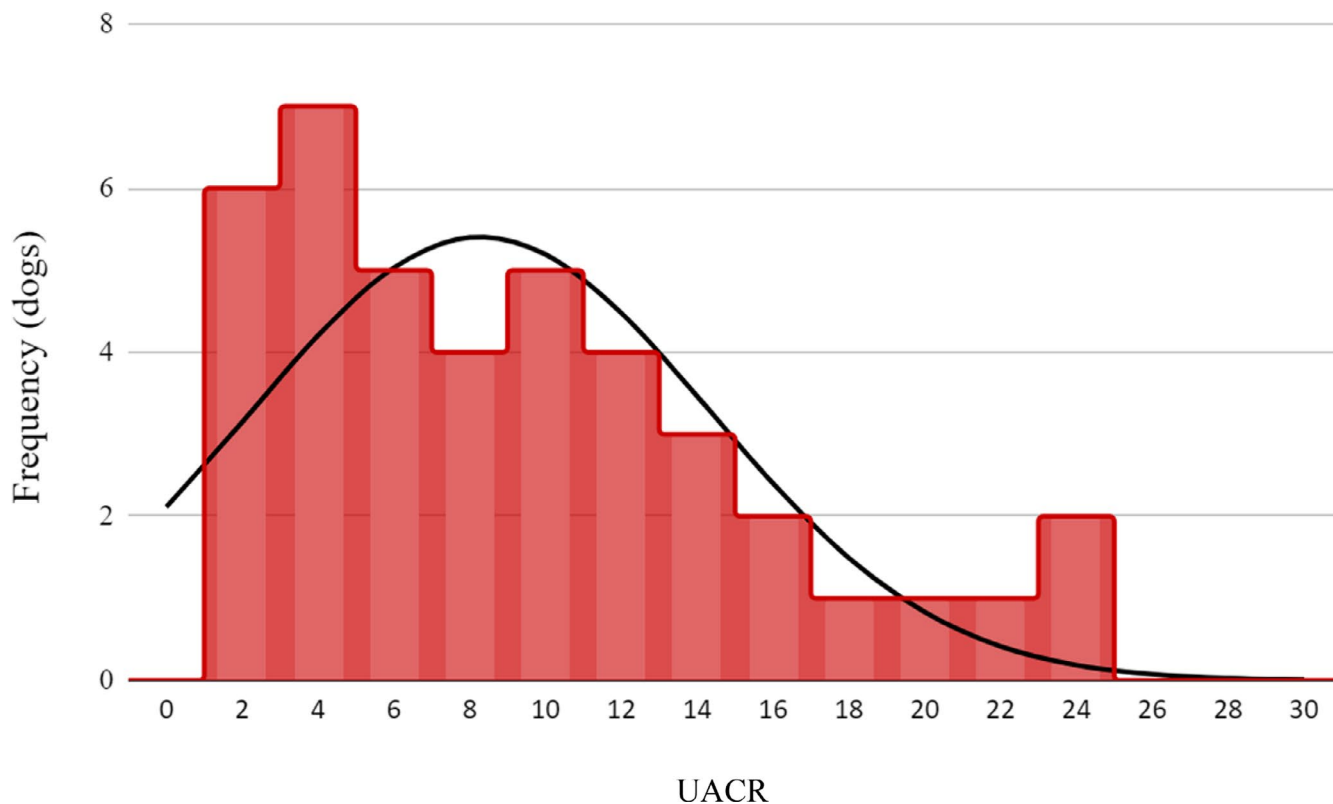
FIGURE 2 Histogram of the urine ammonia-to-creatinine ratio (UACR) for healthy dogs with the overlaid normal distribution. The histogram is derived from the UACR results from our reference population with the outliers removed and is overlaid with a normal distribution. n = 41

highest recorded ammonia values (1013 and 620 mmol/L), while four of the other outliers had the lowest recorded creatinine values (0.49-1.16 mmol/L). Some urinary tract pathogens can produce urease which generates ammonia from urea, which could lead to severely increased urinary ammonia levels. None of the dogs included in the study population had evidence of a urinary tract infection with an active sediment (elevations in RBCs and WBCs), increased urine pH, or observed bacteria. However, urine cultures were not performed, so the possibility of subclinical infection cannot be excluded.

There are several limitations to this study. Attempts to exclude animals with diseases impacting ammonia excretion were based on the history, physical examination, and limited laboratory findings. Further assessment of individual health with the glomerular filtration rate measurement, blood pressure assessment, renal biopsies, and hematologic testing was not performed. It is possible that some dogs had subclinical disease, but efforts to minimize this effect were made using the ASVCP guidelines to calculate a de novo RI.[12] Other limitations of this study were the limited data available on the muscle condition score for the reference population. Lean body mass and age have been found to contribute significantly to serum creatinine concentration which limits the utility of this biomarker for monitoring kidney function in dogs of increasing age and decreasing lean body mass. Unfortunately, during the data collection period, muscle condition score was not a standardized portion of the physical examination, so we are unable to assess the possible impact of lean muscle mass on UAC.

5 | CONCLUSION

This study determined the RI for the UACR in a population of clinically healthy adult dogs. In people, the measurement of urinary ammonia excretion is helpful in determining whether the kidneys are responding appropriately to acid-base challenges.[6,7,13] Further research is warranted to determine whether alterations of UACR are observed in specific disease states in dogs. If so, it is possible that this value could provide useful prognostic information or guide clinical management.

DISCLOSURES

The authors declare no conflict of interest with respect to the research, authorship, and/or publication of this article.

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REFERENCES

1. Weiner ID, Mitch WE, Sands JM. Urea and ammonia metabolism and the control of renal nitrogen excretion. *Clin J Am Soc Nephrol.* 2015;10:1444-1458.
2. Weiner ID, Verlander JW. Ammonia transporters and their role in acid-base balance. *Physiol Rev.* 2017;97:465-494.
3. Mitch WE. Metabolic and clinical consequences of metabolic acidosis. *J Nephrol.* 2006;19(Suppl 9):S70-75.
4. Kraut JA, Madias NE. Metabolic acidosis of CKD: an update. *Am J Kidney Dis.* 2016;67:307-317.
5. Moranne O, Froissart M, Rossert J, et al. Timing of onset of CKD-related metabolic complications. *J Am Soc Nephrol.* 2009;20:164-171.
6. Raphael KL, Carroll DJ, Murray J, et al. Urine ammonium predicts clinical outcomes in hypertensive kidney disease. *J Am Soc Nephrol.* 2017;28:2483-2490.
7. Vallet M, Metzger M, Haymann J-P, et al. Urinary ammonia and long-term outcomes in chronic kidney disease. *Kidney Int.* 2015;88:137-145.
8. Bartges JW. Chronic kidney disease in dogs and cats. *Vet Clin North Am Small Anim Pract.* 2012;42:669-692.
9. Pitts RF, Pilkington LA, MacLeod MB, et al. Metabolism of glutamine by the intact functioning kidney of the dog. Studies in metabolic acidosis and alkalosis. *J Clin Invest.* 1972;51:557-565.
10. Huizenga JR, Tangerman A, Gips CH. Determination of ammonia in biological fluids. *Ann Clin Biochem.* 1994;31:529-543.
11. Harr KE, Flatland B, Nabity M, et al. ASVCP guidelines: allowable total error guidelines for biochemistry. *Vet Clin Pathol.* 2013;42:424-436.
12. Friedrichs KR, Harr KE, Freeman KP, et al. ASVCP reference interval guidelines: determination of de novo reference intervals in veterinary species and other related topics. *Vet Clin Pathol.* 2012;41:441-453.
13. Nagami GT, Hamm LL. Regulation of acid-base balance in chronic kidney disease. *Adv Chronic Kidney Dis.* 2017;24:274-279.
14. Goldstein MB, Bear R, Richardson R, et al. The urine anion gap: a clinically useful index of ammonium excretion. *Am J Med Sci.* 1986;292:198-202.
15. Kim G-H, Han JS, Kim YS, et al. Evaluation of urine acidification by urine anion gap and urine osmolal gap in chronic metabolic acidosis. *Am J Kidney Dis.* 1996;27:42-47.
16. Ha LY, Chiu WW, Davidson JS. Direct urine ammonium measurement: time to discard urine anion and osmolar gaps. *Ann Clin Biochem.* 2012;49:606-608.

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