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Comparison of urinary cortisol, urinary cortisol-to-creatinine ratio, and basal serum cortisol as screening tests for hypoadrenocorticism in dogs

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OBJECTIVE
This study investigates whether urinary cortisol (UC) and UC-to-creatinine ratio (UCCR) perform better than basal serum cortisol (BSC) in identifying dogs with hypoadrenocorticism (HA).

METHODS
A retrospective, multicenter study with 120 client-owned dogs included: 20 with HA, 42 healthy, and 60 with diseases mimicking HA. The UC and UCCR were determined on urine samples using a chemiluminescent enzyme immunoassay. The diagnostic performance of the UC and UCCR were assessed based on receiver operating characteristics (ROC) curves.

RESULTS
A cutoff value of UC < 2 µg/dL revealed 100% sensitivity (95% CI, 83.2 to 100) and 90.0% specificity (95% CI, 79.5 to 96.2) in diagnosing HA. A cutoff value of UCCR < 8.5 X 10^{-6} revealed 100% sensitivity (95% CI, 83.1 to 100) and 71.7% specificity (95% CI, 58.6 to 82.6) in diagnosing HA. A cutoff value of BSC < 2 µg/dL and < 1 µg/dL revealed 100% sensitivity (95% CI, 83.2 to 100) and 51.7% specificity (95% CI, 38.5 to 64.8) and 100% sensitivity (95% CI, 83.9 to 100) and 90% specificity (95% CI, 79.8 to 95.3) in diagnosing HA, respectively.

CONCLUSIONS
BSC < 1 µg/dL showed the same sensitivity but higher specificity than BSC < 2 µg/dL. The UC < 2 µg/dL showed noninferior performance compared with the BSC < 1 µg/dL.

CLINICAL RELEVANCE
UC should be considered a promising screening test for canine HA.

Keywords: urinary cortisol, cortisol, creatinine, basal serum cortisol, hypoadrenocorticism

Hypoadrenocorticism (HA) is an uncommon disease in dogs.[1] Dogs with HA are frequently presented with chronic, unspecific clinical signs, including anorexia, vomiting, weight loss, and diarrhea, while these signs are often vague, waxing and waning, and nonpathopneumonic.[2-5] This is particularly true with eunatremic, eukalemic HA (EEH), also defined as "atypical" HA.[1] A definitive diagnosis of HA requires an ACTH stimulation test (ACTHst).[2] However, the high cost and limited availability of synthetic ACTH in some countries, coupled with the requirement for repeated venipuncture, are some limitations of this test. As a result, basal serum cortisol (BSC) concentration, using a cutoff value of ≥ 2 µg/dL, is commonly used as a screening test to rule out HA. Basal serum cortisol concentration < 2 µg/dL has excellent sensitivity for HA (99.4% to 100%).[6-8] However, due to the low specificity of the test (20% to 78.2%), up to 47% of dogs with gastrointestinal signs but without HA have

Received October 12, 2024
Accepted December 17, 2024
Published online January 21, 2025

doi.org/10.2460/ajvr.24.10.0296

a BSC < 2 µg/dL.[6-12] Two recent studies investigated the urinary cortisol (UC)-to-creatinine ratio (UCCR) as an alternative screening test for HA in dogs.[13] In the study of Del Baldo et al, a UCCR cutoff value of < 4.4 X 10^{-6} yielded 100% sensitivity and 97.3% specificity in diagnosing HA.[13] The study of Moya et al showed even better diagnostic performances.[14] In both studies, UC was measured using a chemiluminescent immunoassay (CLIA; Immulite 2000 cortisol; Siemens Health Care Diagnostics Ltd) validated for dogs.[15] Unfortunately, after analyzing the samples of the 2 above-mentioned studies, there was a change in the Immulite 2000 antibody used for cortisol measurement. An initial review by the European Society of Veterinary Endocrinology—Endocrine Quality Assurance, based on > 40 canine urine results, suggested that the cortisol values measured with the new antibody were lower (average bias, −70%) than the values obtained with the previous antibody (kit before Lot 550).[16] Based on the above findings, the use of the new antibody might result in different diagnostic performances. Therefore, new reference intervals (RIs) and diagnostic performances should be evaluated using the currently available antibody. If the diagnostic performance of UCCR, measuring cortisol with the new antibody, remains good or similar to that observed in previous studies,[13,14] we hypothesize that UCCR and also UC, measured as an absolute value, might be better than BSC in identifying dogs with HA.

This study aims to investigate whether UC and UCCR, used as screening tests, perform better than BSC in identifying dogs with HA.

Methods

Animals and study design

Urine samples collected from privately owned dogs and stored at −20 °C or −80 °C were retrospectively selected from the [University of Bologna Veterinary Teaching Hospital] digital database. The urine samples were collected from January 2020 through March 2023 from dogs with HA or dogs with diseases mimicking HA (DMHA) at the time of diagnosis and routine checkups from the healthy dogs. The protocol was approved by the Scientific Ethics Committee of the [University of Bologna] (no. 57790/2023).

Dogs were included in the HA group if consistent clinical and clinicopathological abnormalities were present and the post-ACTH serum cortisol was ≤ 2 µg/dL. A diagnosis of EEH was made if the following criteria were met: (1) post-ACTH serum cortisol concentration < 2.0 µg/dL, (2) high (> 58 pg/mL) or undetectable (< 5 pg/mL) plasma endogenous ACTH (eACTH) concentrations, and (3) the absence of electrolyte abnormalities. Dogs were excluded from the study if a glucocorticoid medication had been administered within 90 days before testing. Other dogs for which HA was suspected based on clinical signs (vomiting, diarrhea, weakness, lethargy) but were subsequently excluded based on the BSC > 2 µg/dL or ACTHst results (post-ACTH serum cortisol > 5 µg/dL)[17] were included in the DMHA group. Dogs were defined as healthy if no abnormal clinical signs were reported and CBC, serum biochemistry, and urinalysis results were within the RIs.

Sample collection and analytical procedures

The urine samples were collected by free catch (at home or in the hospital) or by ultrasound-guided cystocentesis performed without sedation of the dog. For the ACTHst, blood samples were taken before and 60 minutes after the IV injection of 5 µg/kg synthetic ACTH (Synacthen; Alfasigma SPA). Blood samples for the determination of eACTH concentrations were collected before the injection of synthetic ACTH.

All of the analytical procedures were carried out at the veterinary laboratory of the [Veterinary Teaching Hospital of the University of Bologna]. Blood samples to determine the eACTH were collected into EDTA-coated plastic tubes placed on ice. The samples were immediately centrifuged at 4 °C X 500 *g* for 8 minutes, and the plasma was immediately transferred to plastic tubes, stored at 4 °C, and analyzed within 8 hours or stored at −80 °C and thawed immediately before analysis. Blood samples for the cortisol determination were collected in serum-separating tubes. Clotted blood samples were centrifuged for 10 minutes at 3,000 X *g*; the serum was immediately transferred to plastic tubes, stored at 4 °C, and analyzed the same day or stored at −80 °C and thawed immediately before analysis. Stored urine samples were thawed at room temperature and immediately analyzed on the same day. The serum and UC and eACTH concentrations were measured using a chemiluminescent enzyme immunoassay (Immulite 2000; Siemens Healthcare) validated for dogs and widely used in laboratories worldwide.[15,18,19] Subsequent batches of the kit Lot 550 were used for cortisol analysis. The lower limit of quantification of the assay for cortisol was 1 µg/dL.

The chemistry profile and urine creatinine concentration were measured using an automatic analyzer (AU 480; Beckman Coulter/Olympus). The UCCR was calculated from creatine and cortisol values as previously described.[20] The veterinary laboratory RI for UCCR is between 3.8 X 10^{-6} (90% CI, 3.3 to 4.5) and 22.0 X 10^{-6} (90% CI, 17.9 to 26.9). The RI was calculated using the robust method[21] on urine collected at home from 40 healthy dogs. The cortisol to establish the UCCR RI was measured using the new antibody.

Statistical analysis

Statistical analysis was carried out using commercial statistical software packages (GraphPad Prism, version 7; GraphPad). Data were presented as median and range and analyzed by nonparametric tests. Differences between groups for categorical and numerical variables were analyzed using the Fisher exact test and the Kruskal-Wallis test, respectively. The Kruskal-Wallis test, followed by the Dunn post-test, was carried out to compare the UC, the UCCR, and the BSC from dogs with HA, dogs with DMHA, and healthy dogs. A receiving operating characteristic (ROC) curve was used to determine

the area under the curve and select the optimum UCCR cutoff values to diagnose or exclude HA. The ROC curve analysis was carried out by comparing HA dogs with DMHA. A 95% CI was calculated for the ROC curve. Since the lower limit of quantification of the assay for cortisol was 1 µg/dL, concentrations of serum or UC below 1 µg/dL were reported as 1 µg/dL. The level of significance was set at $P < .05$.

Results

Animals

Twenty dogs with HA were included. Their age ranged from 1 to 13 years (median, 5.5 years), and their body weight ranged from 5.0 to 40.9 kg (median, 17.0 kg). There were 9 males (4 castrated) and 11 females (9 spayed). The HA group consisted of 10 purebred dogs (2 Jack Russell Terriers, 1 English Setter, 1 German Shepherd, 1 Standard Poodle, 1 Rottweiler, 1 Doberman Pinscher, 1 Abruzzese Maremma Shepherd, 1 Italian Spitz, and 1 Iberian Podenco) and 10 mixed-breed dogs. Nineteen dogs had a primary HA, and 1 had a secondary (due to ACTH deficiency) HA. Six dogs had EEHA.

Sixty dogs with DMHA were included. Their age ranged from 0.5 to 15 years (median, 4 years), and their body weight ranged from 3.6 to 50.0 kg (median, 19.8 kg). There were 36 males (4 castrated) and 24 females (9 spayed). Mixed breeds (n = 11) were most common, followed by Labrador Retrievers (n = 4), Miniature Poodles (4), Maltese dogs (4), German Shepherds (n = 3), Border Collies (n = 3), and 31 other purebred dogs for a total of 29 different breeds. The final diagnoses were chronic enteropathy (53), acute gastroenteritis (5), and megaesophagus (2).

Forty-two healthy dogs were included. Their age ranged from 1 to 15 years (median, 4 years), and their body weight ranged from 2.8 to 49.0 kg (median, 27.0 kg). There were 18 males (5 castrated)

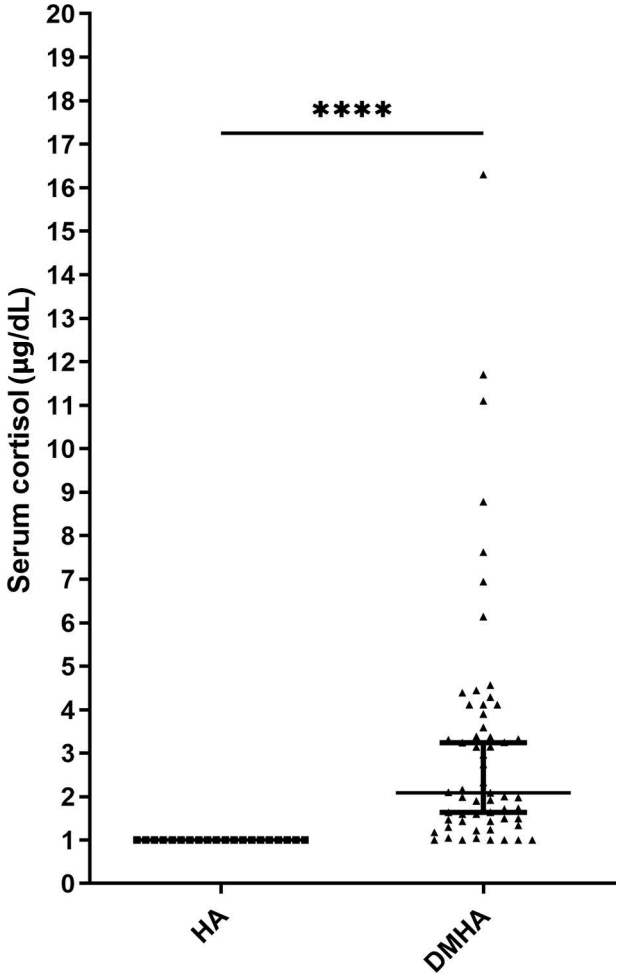

Figure 1—Scatter scale plot comparing basal serum cortisol (BSC) of dogs with hypoadrenocorticism (HA; n = 20) and dogs with disease-mimicking HA (DMHA; n = 60). The horizontal bars represent the median and the 95% CIs. ****$P < .0001$.

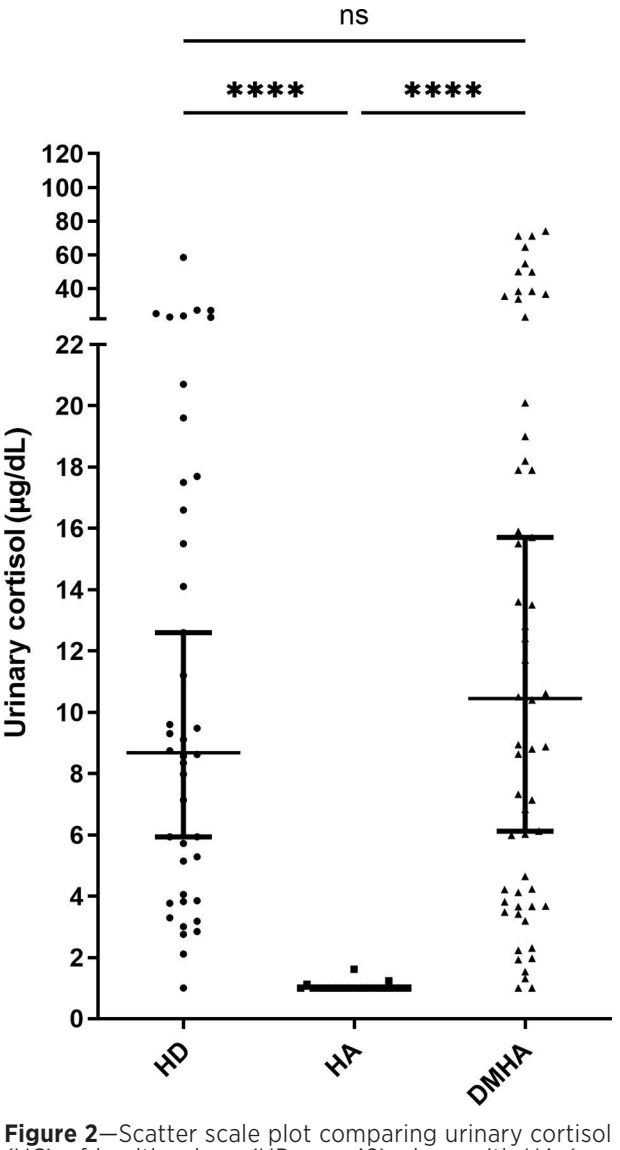

Figure 2—Scatter scale plot comparing urinary cortisol (UC) of healthy dogs (HD; n = 42), dogs with HA (n = 20), and dogs with DMHA (n = 60). Two data points in the DMHA group are outside the axis limit. The horizontal bars represent the median and the 95% CIs. ****$P < .0001$. ns = Not significant.

and 24 females (12 spayed). Mixed breeds (n = 18) were most common, followed by Labrador Retrievers (n = 5), Golden Retrievers (3), German Shepherds (n = 3), and 13 other purebred dogs for a total of 16 different breeds.

There were no significant differences between groups for age, breed distribution, and body weight; spayed females were more represented in the HA group and intact males in the DMHA group.

In the group of dogs with HA, 14 of 20 had hyponatremic and/or hyperkalemic HA, and 6 of 20 had EEH. In dogs with EEH, 5 had high eACTH concentrations (median, 843 pg/mL [258 to 1,134]), and 1 had eACTH < 5 pg/mL.

Basal serum cortisol

The median BSC (µg/dL) was 1.0 (1.0 to 1.0) and 2.1 (1.0 to 16.3) in dogs with HA and dogs with DMHA, respectively. The BSC was below 2 µg/dL in 20 of 20 dogs with HA (100%) and in 28 of 60 dogs with DMHA (46.7%), respectively. The BSC was significantly lower (P < .0001) in dogs with HA than in DMHA **(Figure 1)**. The area under the ROC curve to discriminate dogs with HA from DMHA was 0.95 (95% CI, 0.90 to 0.99). A cutoff value of BSC < 2 µg/dL revealed 100% sensitivity (95% CI, 83.2 to 100) and 51.1% specificity (95% CI, 38.5 to 64.8) in diagnosing HA. A cutoff value of BSC ≤ 1 µg/dL revealed 100% sensitivity (95% CI, 83.9 to 100) and 90.0% specificity (95% CI, 79.8 to 95.3) in diagnosing HA.

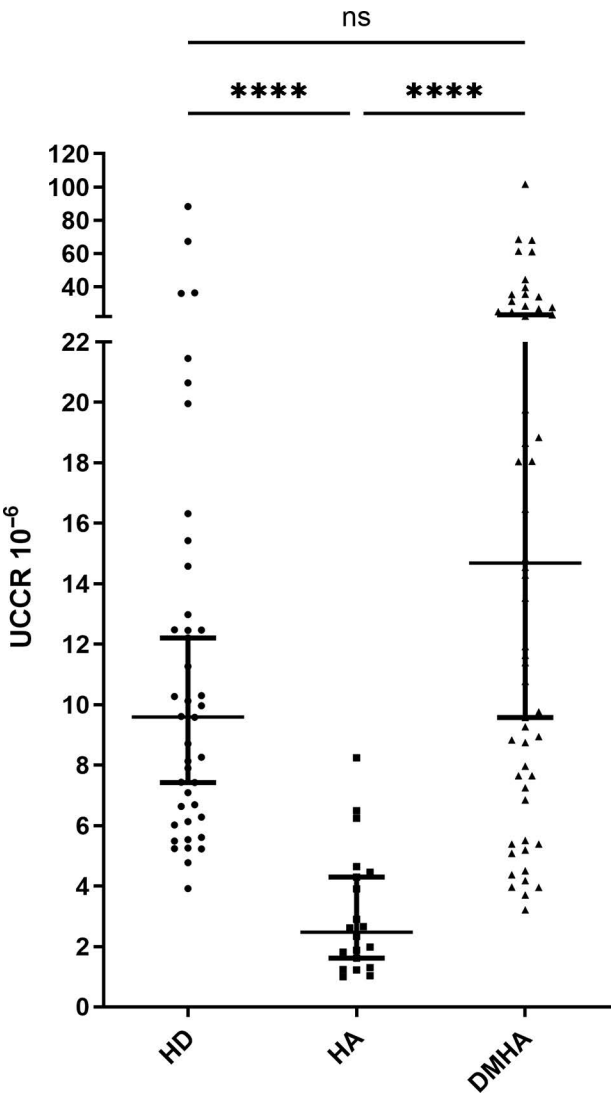

Figure 3—Scatter scale plot comparing UC-to-creatinine ratio (UCCR) of HD (n = 42), dogs with HA (n = 20), and dogs with DMHA (n = 60). The horizontal bars represent the median and the 95% CIs. ****P < .0001.

Urinary cortisol

The median UC (µg/dL) was 8.7 (1.0 to 58.5), 1.0 (1.0 to 1.6), and 10.4 (1.0 to 293.0) in HD, dogs with HA, and dogs with DMHA, respectively. The UC was below 2 µg/dL in 1 of 42 HD (2.4%), 20 of 20 dogs with HA (100%), and 6 of 60 dogs with DMHA (10.0%). The UC was significantly lower (P < .0001) in dogs with HA as compared to HD and DMHA, but there was no significant difference between HD and DMHA **(Figure 2)**. The area under the ROC curve to discriminate dogs with HA from DMHA was 0.98 (95% CI, 0.95 to 1.00). A cutoff value of UC < 2 µg/dL revealed 100% sensitivity (95% CI, 83.2 to 100) and 90.0% specificity (95% CI, 79.5 to 96.2) in diagnosing HA.

Urine cortisol-to-creatinine ratio

The median UCCR was 9.6 X 10^{-6} (3.9 to 88.2 X 10^{-6}), 2.5 (1.0 to 8.2 X 10^{-6}), and 14.7 X 10^{-6} (3.2 to 401.7 X 10^{-6}) in HD, dogs with HA, and dogs with DMHA, respectively. The UCCR was significantly lower (P < .0001) in dogs with HA as compared to HD and DMHA, but there was no significant difference between HD and DMHA **(Figure 3)**. The area under the ROC curve to discriminate dogs with HA from DMHA was 0.95 (95% CI, 0.90 to 0.99; **Figure 4**).

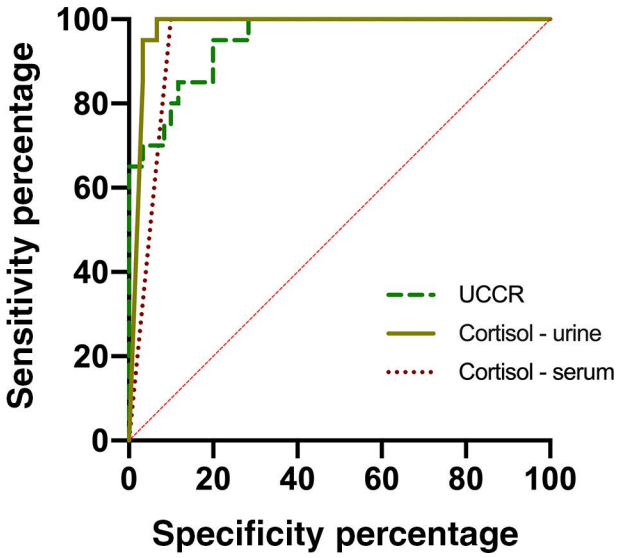

Figure 4—Receiver operating characteristic curves assessing BSC, UC, and UCCR to discriminate dogs with HA from dogs with DMHA. The area under the curve was 0.95 (95% CI, 0.90 to 0.99) for BSC, 0.98 (95% CI, 0.95 to 1.00) for UC, and 0.95 (95% CI, 0.90 to 0.99) for UCCR.

A cutoff value of UCCR < 8.5 X 10^{-6} revealed 100% sensitivity (95% CI, 83.1 to 100) and 71.7% specificity (95% CI, 58.6 to 82.6) in diagnosing HA.

Discussion

This study aimed to evaluate the performances of UC and UCCR as screening tests for HA in dogs, comparing their performances with the commonly used BSC test. The results indicate that UC demonstrates sensitivity and specificity comparable to BSC (when using a cutoff value for BSC of < 1 µg/dL) in identifying dogs with HA.

In this study, the specificity of the BSC in detecting dogs with HA, using the currently recommended cutoff of < 2 µg/dL, was 51.1%, a lower value than that found in other studies.[6–12] Such a lower performance of the BSC is presumably related to the fact that the new antibody of CLIA-Immulite for cortisol measurement was used in this study. It has been reported that the new antibody, compared to the previous one, underestimates cortisol not only in urine but also in serum.[16] Such underestimation would explain why many cases of DMHA showed a BSC value < 2 µg/dL. However, no dogs with HA showed a BSC > 1 µg/dL. Therefore, using the CLIA method with the new antibody, the cutoff value of BSC for ruling out HA should probably no longer be ≥ 2 µg/dL but > 1 µg/dL. However, this study did not aim to establish new BSC cutoffs to investigate HA. Further studies with a larger population of dogs with HA are needed to confirm this finding.

In a previous study,[13] a cutoff value of UCCR < 4.4 X 10^{-6} revealed 100% sensitivity (95% CI, 69.1 to 100) and 97.3% specificity (95% CI, 85.8 to 99.9) in detecting HA, and in another study[14] UCCR ≤ 10.0 X 10^{-6} revealed 100% sensitivity (95% CI, 84.6 to 100) and 100% specificity (95% CI, 95.9 to 100) in detecting HA.[13] In our study, similar to what was observed with the BSC, the UCCR showed a lower specificity than those reported in the above-mentioned studies. This result can be explained by the use of the new antibody, which, by providing lower cortisol results, causes a higher overlap of the UCCR between dogs with HA and DMHA. Despite the lower diagnostic performances with the new antibody, the UCCR showed a higher specificity than the BSC when using the cutoff value of BSC < 2 µg/dL and can still be considered a possible screening test for HA in dogs.

Urinary cortisol with a cutoff of 2 µg/dL and BSC with a cutoff of 1 µg/dL showed the best performance in discriminating dogs with HA from DMHA. An advantage of using UC as an alternative screening test for canine HA is that collecting urine through spontaneous urination is less invasive than blood sampling. A limitation in considering UC as an absolute value is related to the fact that urine concentration influences the concentration of any urinary analyte. In turn, urine concentration can vary significantly due to factors such as hydration status and renal function. For example, in humans, significantly lower urinary free cortisol values are observed in individuals with moderate-to-severe renal impairment because UC excretion is proportional to glomerular filtration rate.[22] This could affect the specificity of UC in dogs with renal impairment screened for HA, resulting in falsely low UC concentrations. Moreover, high daily fluid intake can result in higher cortisol excretion rates due to increased urine volumes that reduce the fraction of filtered cortisol metabolized or reabsorbed in the kidney.[23,24] By relating cortisol to creatinine, which is excreted at a relatively constant rate, these variations can be corrected, providing a better reflection of cortisol excretion. Nonetheless, UCCR had lower specificity when compared to UC in the present study. This finding could be related to the limit of detection of the cortisol assay; indeed, a urine cortisol concentration below 1 µg/dL was reported as equal to 1 µg/dL, thus falsely increasing the UCCR in dogs with very low urine creatinine concentration and negatively affecting the UCCR specificity to discriminate dogs with HA and dogs with DMHA. The chemiluminescent enzyme immunoassay used in this study to measure serum and urine cortisol is capable of quantifying concentrations of cortisol < 1 µg/dL. However, the manufacturer does not guarantee linearity below 1 µg/dL. Therefore, in this study, values < 1 µg/dL were considered as 1 µg/dL.

However, this study was conducted using dogs with HA and DMHA without selecting them based on urinary concentration; therefore, it reflects the real condition of the clinical setting. Also, urine-specific gravity was not available in all dogs.

Future studies that will also evaluate the influence of urine concentration and renal function may clarify whether the latter may have a significant impact on UC diagnostic performances. However, it is important to underline that UC is a screening test, not a confirmatory test. The suspicion of HA due to a low UC value must then be confirmed with the ACTHst. Therefore, the evaluation of DMHA dogs with very dilute urine would likely result in more dogs with UC < 2 µg/dL; this might lower the specificity of the test but would not cause overdiagnosis of HA.

Another limitation of the present study is that both urine collected at home and at the hospital were used, and this potentially affected our results. Previous studies[25] observed significantly increased UCCRs if urine was taken in the hospital compared to at home. Therefore, urine samples for UCCR measurement in the diagnosis of Cushing syndrome should be collected in the dog's home environment to avoid the influence of stress on glucocorticoid secretion. The authors of the present study hypothesize that when screening for HA, it would be most appropriate to collect all urine in the hospital as this would maximize stress and likely allow for further separation of HA and DMHA. A previous study[26] in dogs found significantly increased UCCRs if urine was taken in the hospital compared to at home. Indeed, a stressful situation, such as that encountered in a hospital setting, would likely cause an elevation in blood and UC in dogs without HA but not in those with HA.

Other limitations of this study are the low number of dogs included and the lack of BSC measurement in healthy dogs, which could provide a baseline for comparison. Additionally, the study relied on stored urine samples, which may introduce variability despite consistent storage conditions.

In conclusion, the study demonstrates that UC and, to a lesser extent, UCCR are promising alternatives to BSC for the initial screening of HA in dogs. The high sensitivity ensures that HA cases are not missed, and the higher specificity compared to BSC performed using the current cutoff (2 µg/dL) means fewer dogs will undergo unnecessary further testing or treatment. The BSC remains an adequate screening test as long as a lower cutoff (1 µg/dL) is used; however, studies including large numbers of dogs with HA will be needed to confirm the validity of this possible new cutoff.

Acknowledgments

None reported.

Disclosures

The authors have nothing to disclose. No AI-assisted technologies were used in the generation of this manuscript.

Funding

This study was supported by the European College of Veterinary Internal Medicine Clinical Studies Fund (European Society of Veterinary Endocrinology).

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