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Urinary cortisol-to-creatinine ratio using a chemiluminescent assay has limited diagnostic accuracy for canine hypercortisolism

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Objective
To establish the de novo reference interval (RI) for urinary cortisol-to-creatinine ratio (UCCR) in healthy dogs (HDs) using the currently available chemiluminescent enzyme immunoassay antibody (Veterinary Cortisol; IMMULITE 2000 XPi; Siemens Healthineers) and to evaluate UCCR diagnostic performance in dogs with hypercortisolism (HC).

Methods
This was a retrospective, single-center, observational study. Stored urine samples from dogs with HC or diseases mimicking HC (DMHC) at the time of diagnosis were selected from July 2019 through November 2022. Healthy dogs were prospectively included. The diagnostic performance of the UCCR was assessed based on receiver operating characteristic curves.

Results
One hundred seventy-two dogs were included: 97 with HC, 35 with DMHC, and 40 HDs. The de novo RI for UCCR in HDs was between 3 X 10^{-6} (90% CI, 2.3 X 10^{-6} to 3.8 X 10^{-6}) and 26 X 10^{-6} (90% CI, 29.7 X 10^{-6} to 35.0 X 10^{-6}). The median UCCR was significantly higher in dogs with HC (70.9 X 10^{-6}; 6.8 X 10^{-6} to 882.2 X 10^{-6}) as compared to dogs with DMHC (15 X 10^{-6}; 2.63 X 10^{-6} to 137.8 X 10^{-6}) and HDs (9.1 X 10^{-6}; 3.9 X 10^{-6} to 36.3 X 10^{-6}). The area under the receiver operating characteristic curve for UCCR to differentiate HC dogs from dogs with DMHC was 0.85 (95% CI, 0.78 to 0.92). Using the upper limit of the de novo RI as the cutoff value (UCCR > 26 X 10^{-6}), the sensitivity and the specificity for the UCCR in diagnosing HC were 80.4% (95% CI, 71.1% to 87.8%) and 71.4% (95% CI, 53.7% to 85.4%), respectively.

Conclusions
Using the upper limit of the de novo RI, UCCR showed modest performances not only due to low specificity but also due to a sensitivity of only 80.4%.

Clinical Relevance
UCCR should not be used alone to rule out HC in dogs, and, when the clinical suspicion for HC is present, other endocrine tests should be pursued.

Keywords: urinary cortisol, cortisol, creatinine, Cushing syndrome, UCCR accuracy

Naturally occurring hypercortisolism (HC) is a common endocrine disorder in dogs caused by excessive cortisol secretion from the adrenal cortex. The determination of urinary corticoid (cortisol and its metabolites) excretion is an accepted screening test in the diagnosis of HC, and the urine corticoid-to-creatinine ratio (UCCR) in a random urine sample is expected to be substantially increased in dogs with HC compared to healthy dogs. The UCCR provides an integrated measurement of corticoid production over a given interval, thereby overcoming the problem of fluctuations in plasma concentrations.[1] The greatest advantage is the need for only a single urine sample. Moreover, it is easy to carry out and relatively economical. The diagnostic sensitivity of the test is generally high,[1–8] while its specificity shows greater variability, ranging from 21% to 100%.[1–8] For this reason, this has historically always been considered a good test to rule out the diagnosis of HC but not to rule it in.[9] Several preanalytical and analytical factors are known to influence the diagnostic accuracy

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of the UCCR.[10,11] Elevated UCCR values have been documented not only in dogs with HC but also in those with nonadrenal illnesses[3,4,7,8] and in healthy dogs exposed to stressful conditions, such as hospitalization or veterinary procedures.[11–13] The method of urine collection (free catch vs cystocentesis/catheterization; at home [AH] vs in hospital [IH]) may also influence UCCR specificity.[11,14] The diagnostic performance of the UCCR is further influenced by the assay used to measure the urine cortisol concentration. Over the years, multiple methods, including radioimmunoassay and chemiluminescent immunoassays (CLIAs), have been employed in studies[1–8] investigating the UCCR. Currently, the most popular assay in veterinary laboratories is the automated CLIA. While this method has largely replaced radioimmunoassay, data on its diagnostic performance remain limited. Studies[7,15] using this method reported sensitivities in detecting HC between 86% and 97%. In 2020, the introduction of a new antibody used for cortisol measurement in the IMMULITE 2000 system changed the performances of the assay.[16] An initial review by the European Society of Veterinary Endocrinology–Endocrine Quality Assurance, based on over 40 canine urine samples, revealed that the new antibody produced significantly lower cortisol values (average bias, −70%) compared to the previous assay (kits prior to Lot 550).[16] This methodological change likely impacts the diagnostic performance of the UCCR. As a result, it is essential to establish an updated reference interval (RI; de novo RI) and reassess the diagnostic utility of the UCCR using the currently available antibody. To date, only 1 study[8] has evaluated the diagnostic performances of the UCCR to detect HC with subsequent batches of the kit Lot 550. However, that study included only 12 dogs with HC, and the control group primarily consisted of dogs with other diseases that did not mimic HC, with only 16 dogs with diseases mimicking HC (DMHC). Therefore, additional studies are needed to address these limitations. With this background, this study aimed to establish a de novo RI for UCCR in healthy dogs using the currently available CLIA antibody (Veterinary Cortisol; IMMULITE 2000 XPi; Siemens Healthineers) and to evaluate UCCR diagnostic performance in dogs with HC.

Methods

Animals and study design

Urine samples stored at −20 or −80 °C from privately owned dogs were retrospectively selected from the University of Bologna Veterinary Teaching Hospital digital database. The urine samples were collected from July 2019 through November 2022 from dogs with HC or DMHC at the time of diagnosis. The protocol was approved by the Scientific Ethics Committee of the University of Bologna.

The diagnosis of HC was based on a combination of history (eg, polyuria and polydipsia and polyphagia and dermatological alterations), physical examination findings (eg, alopecia and abdominal enlargement), hematology (eg, lymphopenia, neutrophilia, and thrombocytosis), biochemistry (eg, abnormally high ALT, ALP, and GGT), urinalysis (eg, low urine specific gravity and proteinuria), and endocrine testing (low-dose dexamethasone suppression test and ACTH stimulation test).[17] A diagnosis of pituitary-dependent HC (PDH) was made if any of the following criteria were met: a normal or high concentration of plasma endogenous ACTH (eACTH) concentration (> 5 pg/mL), a cortisol concentration 8 hours after dexamethasone suppression above the lower limit of detection of the assay (1.4 µg/dL) and a cortisol concentration 4 hours after dexamethasone suppression below the lower limit of detection of the assay (1 µg/dL or 28 nmol/L) or less than 50% baseline, pituitary enlargement on MRI or CT (pituitary height-to-brain value > 0.31 X 10^{-2} mm^{-1}),[18] or ultrasonographically bilaterally symmetric normal-sized or enlarged adrenal glands (width > 7.5 mm if breed-specific cutoffs were not available).[17] A diagnosis of adrenal-dependent HC (ADH) was made if the following criteria were met: low or undetectable eACTH (≤ 5 pg/mL) and an ultrasonographically observed unilateral adrenal enlargement with atrophy of the contralateral adrenal gland. A diagnosis of concurrent PDH and the adrenal tumor was made if there was pituitary enlargement on CT or MRI, nonsuppressed eACTH (> 5 pg/mL), and the presence of an asymmetrically enlarged adrenal gland on CT or MRI with the contralateral gland within the normal limit.[17,19]

Dogs in which HC was initially suspected based on clinical signs but subsequently excluded through a low-dose dexamethasone suppression test (LDDST; T8 < 1.4 µg/dL) or an ACTH stimulation test (post-ACTH serum cortisol < 17 µg/dL) were included in the DMHC group. Additionally, this group included dogs with adrenal masses (with or without clinical signs suggestive of HC) in which HC was excluded based on LDDST (T8 < 1.4 µg/dL) and eACTH measurement (eACTH > 10 pg/mL).

In addition, client- and staff-owned healthy dogs were prospectively included in order to establish the de novo RI for the UCCR and to investigate the influence of stress on the UCCR. Dogs were defined as healthy if no clinical signs were reported and if hematology, serum biochemistry, and urinalysis results were within the RI. Clients who signed an informed consent form were asked to collect a midstream voided urine sample in a urine-specific dry, clean plastic cup on the morning of their appointment (within 3 hours of the appointment time) and to store the sample in a refrigerator until leaving for the hospital. Upon arrival at the hospital, clients submitted the urine samples to investigators. Study dogs completed their scheduled appointments and were taken to an external space to obtain an additional voided urine sample. Healthy dogs that required cystocentesis or urinary catheterization to obtain a urine sample were excluded from the study.

Sample collection and endocrine tests

In dogs with HC and DMHC, the urine samples were collected by free catch (AH or IH) or by ultrasound-guided cystocentesis performed without

sedation of the dog. In healthy dogs, urine samples were prospectively collected by free catch AH and, in 26 of them, IH.

For the ACTH stimulation test, blood samples were taken before and 60 minutes after the IV injection of 5 µg/kg of synthetic ACTH (Synacthen; Alfasigma SpA). For the LDDST, blood samples were taken before and 4 hours and 8 hours after the IV injection of dexamethasone (Dexadreson; MSD Animal Health Srl). Blood samples to determine the eACTH were collected into K_3-EDTA–coated plastic tubes placed on ice. The samples were immediately centrifuged at 4 °C, 500 X *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. Stored urine samples were thawed at room temperature and immediately analyzed on the same day. All of the analytical procedures were carried out at the clinical pathology service of the [masked for review]. Plasma eACTH was measured using a CLIA (IMMULITE 2000 ACTH; Diagnostic Product Corp), which detects the intact ACTH molecule (amino acids 1 through 39) and has been previously validated for use in dogs.[20] The manufacturer's instructions indicated an analytical sensitivity for the assay of 5 pg/mL and a calibration range of up to 1,250 pg/mL.[21] The serum and urinary cortisol were measured using a CLIA (Veterinary Cortisol; IMMULITE 2000 XPi; Siemens Healthineers) validated for dogs and widely used in laboratories worldwide.[20,22,23] 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 specific methods on an automated chemistry analyzer (AU 480; Creatinine OSR6178; Beckman Coulter), with urine creatinine determined by the Jaffé kinetic method. The UCCR was calculated from urinary creatine and cortisol values as previously described.[1]

Statistical analysis

Statistical analysis was carried out using commercial statistical software packages (Prism, version 7; GraphPad Software Inc; and MedCalc Statistical Software version 19.4; MedCalc Software Ltd., Ostend, Belgium). Data were presented as median and range and analyzed with nonparametric tests. The de novo RI for the UCCR was established on urine collected AH from the 40 healthy dogs using the robust method according to the American Society for Veterinary Clinical Pathology guidelines.[24] The UCCRs of home and hospital sample pairs were compared using the Mann-Whitney *U* test.

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 hoc test, was carried out to compare the UCCR from dogs with HC, dogs with DMHC, and healthy dogs. In healthy dogs, the UCCR from urine samples collected AH was used for comparison. The diagnostic utility of the UCCR to differentiate between dogs with HC and dogs with DMHC was assessed by receiver operating characteristic (ROC) curves. A 95% CI was calculated for the ROC curve. Sensitivity, specificity, positive predictive values, and negative predictive values (NPVs) of the UCCR were also calculated. The level of significance was set at $P < .05$.

Results

Animals

Ninety-seven dogs with HC were included. Forty-six of them were male (15 castrated) and 50 female (42 spayed). Their median body weight was 11.2 kg (3.2 to 51.7), and their median age was 11.2 years (5.3 to 17.1). The HC group consisted of 59 purebred dogs (7 Dachshunds, 5 Maltese, 4 Jack Russel Terriers, 3 Fox Terriers, 3 Galgos Españoles, 3 Shih Tzu, 3 Yorkshire Terriers, 3 Miniature Poodles, 2 Boston Terriers, 2 French Bulldogs, 2 Chihuahuas, 2 pit bull–type dogs, 2 Golden Retrievers, 2 Labrador Retrievers, 2 Lagotto Romagnolo dogs, 1 Basset Hound, 1 Bolognese, 1 Boxer, 1 English Bulldog, 1 Cavalier King Charles Spaniel, 1 Cairn Terrier, 1 Cocker Spaniel, 1 Greyhound, 1 Griffon Bley de Gascogne, 1 Pinscher, 1 Alaskan Malamute, 1 Siberian Husky, 1 Tibetan Terrier, and 1 Weimaraner) and 38 mixed-breed dogs. Ninety-three of them were diagnosed with PDH, 3 with ADH, and 1 with concurrent PDH and ADH.

Thirty-five dogs with DMHC were included. Sixteen of them were male (7 castrated) and 19 female (14 spayed). Their median body weight was 14.4 kg (3 to 45.8), and their median age was 12.7 years (7.3 to 18.3). This group consisted of 15 purebred dogs (4 Labrador Retrievers, 1 Australian Shepherd, 2 Poodles, 2 Dachshunds, 1 Bolognese, 1 Cane Corso, 2 Jack Russell Terriers, 1 Cavalier King Charles Spaniel, 1 Lagotto Romagnolo, 1 Maltese, 1 Italian Bloodhound, 1 Samoyed, 1 Shih Tzu, and 1 Yorkshire Terrier) and 20 mixed-breed dogs. The final diagnoses were nonsecreting adrenal tumor (13), diabetes mellitus (5), pheochromocytoma (3), nonsecreting pituitary adenoma (2), chronic hepatopathy (2), primary hyperparathyroidism (2), aldosteronoma (1), hypothyroidism (1), psychogenic polydipsia (1), and unknown (5).

Forty healthy dogs were included. Eighteen of them were male (5 castrated) and 22 female (12 spayed). Their median body weight was 27.5 kg (2.8 to 49), and their median age was 4.4 years (1 to 15.9).

This group consisted of 18 mixed-breed dogs and 22 purebred dogs (5 Labrador Retrievers, 3 Golden Retrievers, 3 German Shepherd Dogs, 2 Whippets, 1 Border Collie, 1 Boxer, 1 Bernes Mountain dog, 1 Maltese, 1 Leonberger, 1 Chihuahua, 1 Australian Shepherd, 1 Maremma Shepherd, and 1 English Setter).

There were no significant differences between groups for age, sex, and bodyweight.

Urine corticoid-to-creatinine ratio

The de novo RI for the UCCR in healthy dogs was between 3 X 10^{-6} (90% CI, 2.3 to 3.8) and 26 X 10^{-6} (90% CI, 29.7 to 35.0).

The median (minimum to maximum) UCCR was 11.7 X 10^{-6} (5.3 X 10^{-6} to 45.8 X 10^{-6}) for urine samples collected in the IH and 8.19 X 10^{-6} (3.9 X 10^{-6} to 36.3 X 10^{-6}) for urine samples collected AH. The median UCCR was significantly lower (P = .03) in AH samples as compared to IH samples **(Figure 1)**.

The median UCCR was 70.9 X 10^{-6} (6.8 X 10^{-6} to 882.2 X 10^{-6}), 15 X 10^{-6} (2.63 X 10^{-6} to 137.8 X 10^{-6}), and 9.1 X 10^{-6} (3.9 X 10^{-6} to 36.3 X 10^{-6}) in dogs with HC, dogs with DMHC, and healthy dogs, respectively. The median (range) UCCR values in the different categories of DMHC groups is reported in **Table 1**. For categories with only 1 subject, the individual value is reported instead of the median and range. The median UCCR was significantly higher (P < .0001) in the dogs with HC as compared to the dogs with DMHC and healthy dogs **(Figure 2)**. The area under the ROC curve (AUC) for the UCCR to differentiate HC dogs from dogs with DMHC was 0.85 (95% CI, 0.78 to 0.92). Using the upper limit of the de novo RI (UCCR > 26 X 10^{-6}) as the cutoff value, the sensitivity and the specificity for the UCCR in diagnosing HC were 80.4% (95% CI, 71.1% to 87.8%) and 71.4% (95% CI, 53.7% to 85.4%), respectively **(Figure 3)**. The positive predictive values and NPVs were 88.6% and 56.8%, respectively.

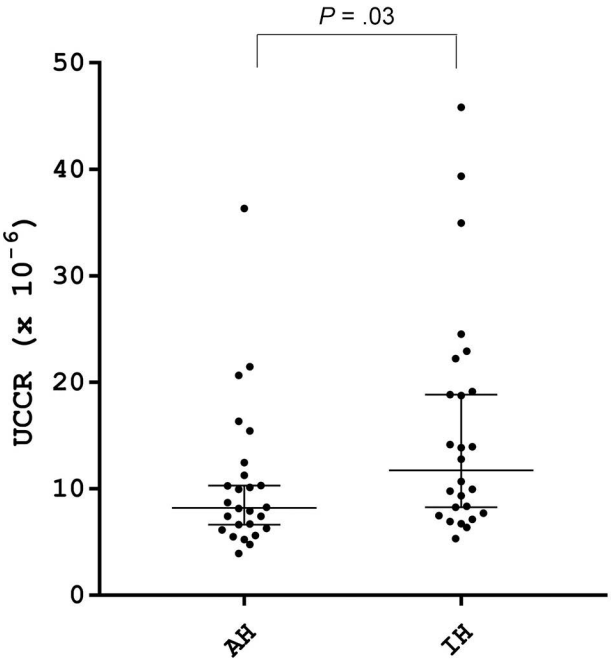

Figure 1—Scatter scale plot comparing urinary cortisol-to-creatinine ratio (UCCR) for urine samples collected in hospital (IH) and those collected at home (AH). The horizontal bars represent the median and the 95% CI.

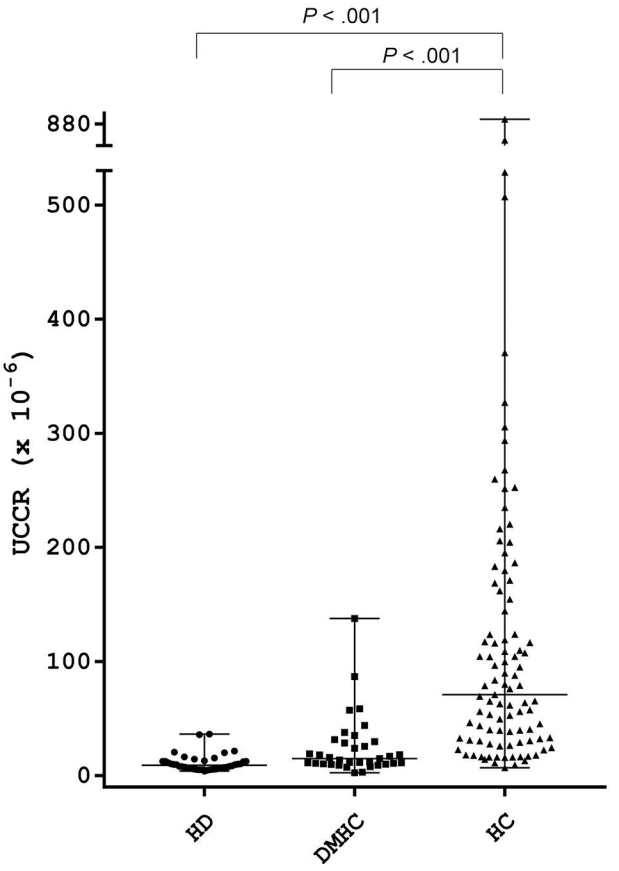

Figure 2—Scatter scale plot comparing UCCR of dogs with hypercortisolism (HC; n = 97), dogs with diseases mimicking HC (DMHC; n = 35), and healthy dogs (HDs; n = 40). The horizontal bars represent the median, the maximum, and the minimum value of each group.

Table 1—The median (range) urinary cortisol-to-creatinine ratio values in the different categories of diseases mimicking hypercortisolism group.

Condition	Median/values	Minimum	Maximum
Nonsecreting adrenal tumors (13)	13.80	10.10	58.65
Diabetes mellitus (5)	15.88	2.92	28.67
Pheochromocytoma (3)	37.86	10.81	137.80
Nonsecreting pituitary adenoma (2)	11.22	7.40	15.04
Chronic hepatopathy (2)	14.14	9.84	18.43
Primary hyperparathyroidism (2)	14.20	9.22	19.17
Aldosteronoma (1)	9.10	—	—
Hypothyroidism (1)	86.91	—	—
Psychogenic polydipsia (1)	2.63	—	—
Unknown (5)	17.01	11.20	25.64

For categories with only 1 subject, the individual value is reported instead of the median and range.

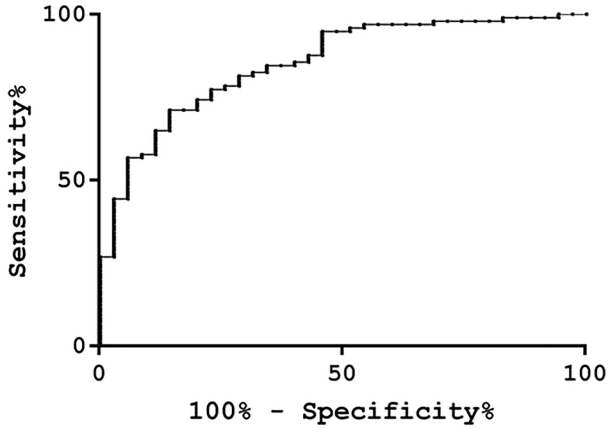

Figure 3—Receiver operating characteristic curves for the use of UCCR to differentiate dogs with HC from dogs with DMHC.

Discussion

In the current study, we established a de novo RI of the UCCR using the CLIA (Veterinary Cortisol; Immulite 2000 XPi; Siemens Healthineers) and reevaluated the diagnostic performances of thee UCCR for the diagnosis of HC.

The de novo RI for the UCCR calculated from 40 healthy dogs was between 3 X 10^{-6} (90% CI, 2.3 X 10^{-6} to 3.8 X 10^{-6}) and 26 X 10^{-6} (90% CI, 29.7 X 10^{-6} to 35.0 X 10^{-6}). The upper limit of the RI is higher than that obtained in a recent study[8] that used the same CLIA IMMULITE 2000 XPi (Veterinary Cortisol; IMMULITE 2000 XPi; Siemens Healthineers; 19.8 X 10^{-6}) and lower than the RI determined by a previous study[7] (30.8 X 10^{-6}) using the CLIA IMMULITE 1000 (Cortisol; IMMULITE 1000; Siemens Healthineers). The difference in analytical methods used for urinary cortisol measurement may explain the lower values recorded with the IMMULITE 2000 XPi compared to the IMMULITE 1000. Indeed, the British Small Animal Veterinary Association and the European Society of Veterinary Endocrinology indicated that the cortisol assay using the IMMULITE 2000 showed lower values than previously measured due to changes in antibodies.[16] The difference between the upper limit of the RI obtained in this study (26 X 10^{-6}) and that reported in a previous study (19 X 10^{-6}) using the same IMMULITE 2000 assay could be attributed to variations in the study populations, such as age, breed, or body weight distribution. Additionally, preanalytical factors, such as differences in sample handling (eg, storage and transport conditions), and differences in statistical methods used to calculate the RI, such as the exclusion or inclusion of outliers and sample size differences, may also explain this variability.

In our study, the UCCR was significantly higher in samples collected from healthy dogs at the veterinary hospital compared to the UCCR in samples collected AH. This finding is similar to those of previous studies[8,12,13,25,26] where veterinary care and setting were shown to increase overall stress level. Because the diurnal variation of cortisol excretion in urine is unlikely in dogs,[7,27] the higher values IH were probably due to stress. Thus, it is strongly recommended that UCCR measurements should be performed using free-catch urine collected AH to minimize the impact of stress on glucocorticoid secretion and avoid HC misdiagnosis.

The results of our study showed that dogs with HC have significantly higher UCCR values than dogs with DMHC. However, substantial overlap was found (Figure 2). Using the upper limit of the de novo RI as the cutoff, the AUC of the UCCR for differentiating HC from dogs with DMHC was 0.85, which is lower than previously reported. A previous study[7] using a previous version of the CLIA (Cortisol; IMMULITE 1000; Siemens Healthineers) to measure urinary cortisol concentration showed an AUC of 0.94, whereas a recent study[8] that used the same CLIA (Veterinary Cortisol; IMMULITE 2000 XPi; Siemens Healthineers) and antibody evaluated in our study showed an AUC of 0.97. The differences in diagnostic performance despite the use of the same CLIA IMMULITE 2000 with the same antibody could be attributed to several factors. First, the population size and composition varied significantly. Our study included a larger and more diverse population, with 97 dogs with HC and 35 with DMHC, compared to the smaller cohort of 12 dogs with HC and 16 with DMHC in the study by Nagata et al.[8] A larger sample size, while providing a more robust evaluation, also introduces greater biological variability and a wider spectrum of disease severity, which may reduce the discriminatory power of UCCR. Moreover, our study likely encompassed a broader range of clinical presentations, including milder or early-stage cases of HC, which may have UCCR values closer to those seen in DMHC, contributing to the observed overlap. In contrast, the smaller sample in the previous study might have included more "classic" or severe cases of HC, which are easier to distinguish and may have artificially inflated diagnostic performance metrics, such as AUC and sensitivity. Finally, methodological factors could also play a role. Despite using the same analytical method (Veterinary Cortisol; IMMULITE 2000 XPi; Siemens Healthineers) with the same antibody, differences in laboratory procedures, sample handling, or environmental stressors during urine collection could influence cortisol concentrations and, consequently, UCCR values. These findings highlight the challenges of using a single UCCR result for differentiating HC from DMHC in the clinical setting and emphasize the need for cautious interpretation of UCCR values, particularly in dogs with mild clinical signs. Using the upper limit of the de novo RI as the cutoff, the diagnostic sensitivity of the assay was lower (80%) compared to that obtained in previous studies using the IMMULITE 1000 (Cortisol; IMMULITE 1000; Siemens Healthineers; 97% sensitivity)[7] or the IMMULITE 2000 (Veterinary Cortisol; IMMULITE 2000 XPi; Siemens Healthineers) with the same antibody (91.7% sensitivity),[8] indicating that the UCCR cannot be used alone to rule out HC. This is also highlighted by the low NPV of 56.8%, which suggests that a UCCR below this cutoff does not reliably exclude the disease. The UCCR result within the RI

cannot exclude HC in dogs because UCCR principally assesses cortisol production but not adrenocortical reserve or resistance to glucocorticoid feedback.[16] In cases with clinical suspicion of HC and UCCR within RI, other endocrine tests, such as an ACTH stimulation test and LDDST, should be performed.

The specificity using the upper limit of the de novo RI as the cutoff was 71%, which is similar to those obtained in previous studies using the CLIA method IMMULITE 1000 (Cortisol; IMMULITE 1000; Siemens Healthineers; 68%[7] and 63%[15] specificity) or IMMULITE 2000 (Veterinary Cortisol; IMMULITE 2000 XPi; Siemens Healthineers) with the same antibody (75% specificity)[8] but is lower compared to the specificity reported in other studies[1,3–5] in which a different assay was used. Differences between control groups (ie, healthy or affected by DMHC) and urine sampling method (ie, free catch vs cystocentesis and AH vs IH) can affect the diagnostic test specificity. Indeed, the highest specificity (from > 95% to 100%) was documented using control groups comprised of healthy dogs or dogs with nonadrenal illness in which HC was not clinically suspected.[1,3–5] Such control populations are unlikely to reflect the type of dogs tested in clinical practice. A lower specificity (ranging from 63% to 85%) was reported in dogs in which HC was considered as a possible differential as in our study[2, 6–8, 15] or when urine was collected in hospitalized dogs.[4,5]

This study has several limitations, primarily related to its retrospective design for the HC and DMHC groups. Urine collection methods were not available for all dogs in these groups. Although it is standard practice at our institution to ask owners to bring in urine samples collected AH, some dogs in the HC and DMHC groups may have had urine samples collected IH (either by free catch or cystocentesis), which could have led to higher UCCR values due to stress-related influences.

It is likely that if all urine samples from dogs with HC had been collected AH, we would have observed an even lower sensitivity due to the potential reduction in stress-related increases in the UCCR. Additionally, follow-up data were not available for all dogs, meaning that the response to treatment was not always recorded in the medical records. As such, some dogs classified as having HC may have been misdiagnosed. Likewise, we cannot exclude the possibility that some dogs in the DMHC group may have actually been affected by HC.

Another limitation of this study is the lack of data on the long-term stability of urinary corticoids at −20 or −80 °C. To our knowledge, no studies have specifically assessed their stability over several years, which may introduce potential variability in the measurements.

A potential limitation of this study is the establishment of the de novo RI using a sample of 40 healthy dogs. While this provides a valuable starting point, a larger sample size (> 120 dogs) could have resulted in a different RI, potentially affecting the CIs of both lower and upper RI limits and, consequently, the diagnostic utility of CLIA-UCCR in distinguishing HC from DMHC. However, recruiting a large cohort of healthy dogs is inherently challenging. Despite this limitation, the RI defined in this study offers relevant insights and contributes to the understanding of CLIA-UCCR as a diagnostic tool.

Finally, this study was conducted in a referral center. Cases of HC presented to a referral center do not necessarily reflect those found in the general population. The preselection of dogs, including preferentially early cases of HC with only mild signs, may have led to less clear test results.

In conclusion, this study identified de novo RI for UCCR in which cortisol was measured by veterinary reagents on the IMMULITE 2000 XPi and confirmed the importance of collecting urine AH to avoid the influence of stress on UCCR results. Moreover, using the upper limit of the de novo RI, the UCCR showed modest performances not only due to low specificity but also due to a sensitivity and NPV of only 80.4% and 56.8%, respectively; therefore, the UCCR should not be used alone to rule out HC in dogs, and, when the clinical suspicion for HC is present, other endocrine tests should be pursued.

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