FULL PAPER

Internal Medicine

Urinary corticoid to creatinine ratios using IMMULITE 2000 XPi for diagnosis of canine hypercortisolism

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ABSTRACT. The urinary corticoid to creatinine ratio (UCCR) is one of the most commonly used screening tests for canine hypercortisolism (HC). In this study, a reference interval was established for UCCR using IMMULITE 2000 XPi, the latest chemiluminescence enzyme immunoassay. The diagnostic performance of this method for UCCR in canine HC was also evaluated. The median UCCR was 1.06×10^{-5} (range: 0.28–2.49) for 58 healthy dogs, and an upper reference limit of 1.98×10^{-5} (90% confidence interval: 1.76–2.15) was determined. The median UCCR in the 12 dogs with HC (7.38×10^{-5}, range 1.86–29.98) was significantly higher than that in the 16 dogs with mimic-HC (1.59×10^{-5}, range 0.47–3.42, $P<0.001$). The area under the curve for UCCR to differentiate HC dogs from mimic-HC dogs was 0.971, with a sensitivity of 91.7% and specificity of 100% when the cut-off value was set at 3.77×10^{-5}. The UCCR of 16 paired urine samples collected at home and in hospital showed that the UCCR of samples collected in the hospital was significantly higher than that of samples collected at home (mean difference 3.30×10^{-5}, 95% confidence interval: 0.70–5.90, $P=0.001$). In summary, we established the upper reference limit for UCCR using IMMULITE 2000 XPi in dogs and confirmed that UCCR is a useful diagnostic test for HC in dogs if urine samples are collected at home.

KEYWORDS: canine, Cushing's syndrome, hyperadrenocorticism, urinary cortisol

Spontaneous canine Cushing's syndrome or hypercortisolism (HC), is one of the most common endocrine diseases in veterinary medicine. An adrenocorticotropic hormone (ACTH)-secreting pituitary tumor, also known as pituitary-dependent HC, is the most common cause of canine HC, accounting for 80–85% of cases [17]. Another major form includes cortisol-secreting adrenocortical tumors, accounting for 15–20% of cases. Rare forms, such as food-dependent HC and ectopic ACTH-secreting tumors, have also been reported [10, 12]. The diagnosis of canine HC is challenging because affected dogs show various types and degrees of clinical signs and there are no perfect diagnostic tests [1]. Commonly used endocrine tests for the diagnosis of HC in dogs include a low-dose dexamethasone suppression test (LDDST), an ACTH stimulation test, and urinary corticoid to creatinine ratios (UCCR) [1]. The UCCR has several advantages over the LDDST and ACTH stimulation test. First, it can be performed without stress because urine is collected at home. Second, it reflects cortisol production without the fluctuations usually observed in serum cortisol measurements. Finally, if used with an oral high-dose dexamethasone suppression test, it can simultaneously assess both hyperproduction of cortisol and resistance to glucocorticoid feedback [1, 11].

Currently, urinary cortisol concentrations are commonly measured using chemiluminescence enzyme immunoassay (CLEIA), although an in-house radioimmunoassay that has little cross-reactivity with other steroid hormones is available in a specific institution [4, 24]. While it is often pointed out that the assay and laboratory-specific reference interval should be established for cortisol measurements, information on such interval values using IMMULITE 2000 XPi, the newest CLEIA method widely used in veterinary medicine, is scarce. In addition, there is no established reference range for measuring UCCR in Japan.

The purpose of this study was to establish an upper reference limit for UCCR using IMMULITE 2000 XPi in a population of healthy dogs in Japan. The second purpose was to evaluate the diagnostic performance of this method for UCCR in canine HC.

MATERIALS AND METHODS

Healthy controls

Client-owned dogs visiting Poplar Animal Hospital, a primary animal hospital in Japan or Hokkaido University Veterinary Teaching Hospital (HUVTH) between February 2021 and October 2021 for health checks were recruited for this study. Residual samples of the supernatant of free-catch urine collected at home by the owners for routine urinalysis were used in this study. Urine was brought to hospital immediately after collection. All dogs were considered healthy based on their medical history, physical examination, complete blood count, serum biochemistry, and urinalysis. None of the dogs received medications that affected cortisol concentrations, such as prednisolone. Informed consent for the collection of residual urine samples was obtained from the owners. Urine samples were stored at −20°C for subsequent measurements. The median time from sample collection to measurement was 117 days (range: 7–243 days).

Clinical cases

Among dogs that visited HUVTH between November 2020 and February 2022, those suspected of having HC and which underwent UCCR measurements using free-catch urine at home were included in the study. Hypercortisolism was suspected on the basis of clinical signs (polyuria/polydipsia, polyphagia, abdominal distension, excessive panting, alopecia, thin skin, or urine leakage), laboratory findings (alkaline phosphatase [ALP] or alanine aminotransferase [ALT] levels above the reference intervals or urine specific gravity <1.020), and/or abdominal ultrasound findings (adrenal enlargements). The following information was collected: signalment, clinical signs, laboratory findings, results of endocrine test (UCCR, LDDST, and ACTH stimulation test), final diagnoses, and response to treatment. Dogs were diagnosed with HC if response to trilostane treatment was confirmed. Dogs were included in the mimic-HC group if they had alternative diagnoses that explained the clinical findings and if 1 of the following criteria was met: (1) clinical signs suggesting HC were improved by the treatment for the diseases or did not progress in at least the 6-month follow-up period, (2) endocrine tests precluded functional adrenocortical tumor in dogs with adrenal mass. For the LDDST and ACTH stimulation test, serum cortisol concentration was measured using enzyme-linked immunosorbent assay (SNAP Cortisol Test Kit, IDEXX Laboratories, Inc., Westbrook, ME, USA) or CLEIA (IMMULITE 1000 or 2000 Cortisol, Siemens Healthineers, Tokyo, Japan). For LDDST, the suppression patterns were determined as described previously [3]. Adrenal mass was confirmed if an apparent nodule or mass was present or dorsoventral thickness was greater than 10 mm in either adrenal gland on ultrasonography [7].

Dogs that visited HUVTH during the same period but were not suspected of having HC were also included in this study as the other disease groups.

None of the dogs received medications that affected cortisol concentrations, such as prednisolone or trilostane.

The owners of the dogs were asked to collect free-catch urine at home in the morning. Urine was brought to the hospital immediately after collection. In some cases, urine was also collected in the hospital by catheterization or cystocentesis when clinicians judged it necessary for clinical purposes. Urine was centrifuged, and the supernatant was immediately subjected to the measurements of UCCR. For urine samples of dogs with other disease groups and those collected in the hospital, the supernatant was stored at −80°C for subsequent measurements. The median time from sample collection to measurement was 37 days (range: 1–289 days).

This study was approved by the Ethics Committee of HUVTH (approval no. 2021-06), and informed consent was obtained from the owner of each dog for urine sample collection.

The measurements of UCCR

Urinary cortisol concentrations were measured with CLEIA (IMMULITE 2000 Veterinary Cortisol) using IMMULITE 2000 XPi (Siemens Healthineers), which has been validated in canine urine samples [18]. The intra-assay coefficient of variation (CV) was analyzed using urine samples with low, middle, and high cortisol concentrations by measuring urine cortisol concentrations 10 times each. The mean concentration of low, middle, and high cortisol concentration was 1.0 µg/dl, 8.5 µg/dl, and 18.1 µg/dl, and the CV was 6.5%, 3.7%, and 3.2%, respectively. The measuring range of urinary cortisol concentration was 0.1–50 µg/dl. Urinary creatinine concentrations were measured by an enzymatic assay (L-Type Creatinine M, FUJIFILM Wako Pure Chemical Corp., Tokyo, Japan) using BioMajesty6050 (JEOL Ltd., Tokyo, Japan). The mean concentration of low, middle, and high creatinine concentration was 15.6 mg/dl, 193.7 mg/dl, and 462.9 mg/dl, and the CV was 0.7%, 0.5%, and 0.2%, respectively. The measured range of urinary creatinine concentration was 0.2–1,000 mg/dl. The UCCR value is represented by the ratio of cortisol concentration (µmol/l) to creatinine concentration (µmol/l). All measurements were performed at a commercial laboratory (FUJIFILM VET Systems, Tokyo, Japan).

Statistical analyses

The reference interval for UCCR was established using a robust parametric method according to the American Society for Veterinary Clinical Pathology [9]. Because the data showed Gaussian distribution, untransformed data were used. This method was performed using a software program (Reference Value Advisor, downloaded from http://www.biostat.envt.fr/reference-value-advisor/) [9, 13]. Other statistical analyses were performed using a commercial software (JMP Pro version 16.0, SAS Institute Inc., Cary, NC, USA). Correlations were assessed using Spearman's rank correlation test. Differences in clinical values between the groups were compared using the Mann–Whitney *U* test or Fisher's exact test. The UCCR among groups was compared using the Kruskal–Wallis test, followed by the Steel–Dwass test. Comparisons of UCCR measurements using different sample collection methods were performed using the Bland–Altman difference plots and Wilcoxon signed-rank test. The receiver operating characteristic curve was generated, and the area under the curve (AUC) was used to evaluate the diagnostic performance of the UCCR. For statistical analysis, values greater than the upper detection limits were defined as the upper value plus 0.1 or 1. Statistical significance was set at $P<0.05$.

RESULTS

Urinary corticoid to creatinine ratios in healthy dogs

Fifty-eight client-owned dogs were included in the study. The median age of these dogs was 4.5 years (range: 5 months–14.7 years) and the median body weight was 5.6 kg (range: 2.1–31.0 kg). Thirty-four dogs were female (33 spayed) and 24 were male (23 neutered). Twenty breeds were included; there was only one dog each of nine breeds, while breeds with more than one dog included mixed breed (n=9), Chihuahua (n=8), Toy poodle (n=8), Miniature Dachshund (n=5), Bichon Frise (n=4), Miniature Schnauzer (n=3), Shiba (n=3), Yorkshire Terrier (n=3), Cavalier King Charles Spaniel (n=2), Labrador Retriever (n=2), and Papillon (n=2).

The median UCCR was 1.06×10^{-5} (range: 0.28–2.49) for healthy dogs. The reference interval of UCCR for this population was established, and an upper reference limit of 1.98×10^{-5} (90% confidence interval: 1.76–2.15) was determined using the robust method. No correlations were found between UCCR and age ($P=0.13$), serum ALT (n=51, $P=0.31$), serum ALP (n=37, $P=0.32$), serum urea (n=52, $P=0.35$), or urine specific gravity ($P=0.63$), whereas there were weak but significant negative correlations between UCCR and body weight ($r=-0.368$, $P=0.005$) and serum creatinine (n=35, $r=-0.365$, $P=0.031$). There was no significant difference in UCCR between female and male dogs ($P=0.87$).

Characteristics of clinical cases

Forty-seven dogs comprising 12 dogs with HC, 16 dogs with mimic-HC, and 19 dogs with other diseases were included. The HC group consisted of 10 dogs with pituitary-dependent HC and two with adrenocortical tumors. The final diagnoses of mimic-HC dogs included five dogs with non-functional adrenal masses with or without chronic kidney disease, three with pheochromocytomas, two with chronic kidney diseases, and one dog each with alopecia X, dietary hyperlipidemia, hypothyroidism, obesity, sudden acquired retinal degeneration syndrome, and urethral sphincter mechanism incompetence. An ACTH stimulation test was performed on 11 HC and 10 mimic-HC dogs. Seven of the 11 dogs with HC showed post-ACTH cortisol concentrations >22 µg/dl, whereas, among the dogs with mimic-HC, five of the 10 dogs showed post-ACTH cortisol concentrations >22 µg/dl. LDDST was performed in five dogs with HC and seven dogs with mimic-HC. Among the five dogs with HC, three showed a lack of suppression patterns and two showed partial suppression patterns. Among the seven dogs with mimic-HC, five showed partial suppression patterns and two showed escape patterns. Other diseases included primary hyperparathyroidism (n=3), hepatocellular carcinoma (n=3), acute kidney injury (n=2), cholangiocellular adenoma (n=1), cholecystitis (n=1), cirrhosis (n=1), diabetes mellitus (n=1), gallbladder mucocele (n=1), osteosarcoma (n=1), protein-losing enteropathy (n=1), protein-losing nephropathy (n=1), pulmonary adenocarcinoma (n=1), splenic tumors (n=1), and systemic metastatic tumors (n=1). There were 20 dog breeds, those with more than one dog were Chihuahua (n=7), mixed breed (n=6), Toy Poodle (n=6), Miniature Dachshund (n=5), Pomeranian (n=5), Bichon Frise (n=2), Shih Tzu (n=2), and West Highland White Terrier (n=2). Twenty-nine were females (25 spayed), and 18 were males (14 neutered).

Age, body weight, sex, plasma urea concentration, plasma creatinine concentration, and urine specific gravity were not significantly different between the HC and mimic-HC groups, whereas plasma ALT activity, plasma ALP activity, urine cortisol concentration, and urine creatinine concentration were significantly different between the groups (Table 1). Dogs with HC showed more frequent abdominal distension and thin skin than dogs with mimic-HC, but the frequencies of polyuria/polydipsia, polyphagia, excessive panting, and alopecia were not significantly different between the groups.
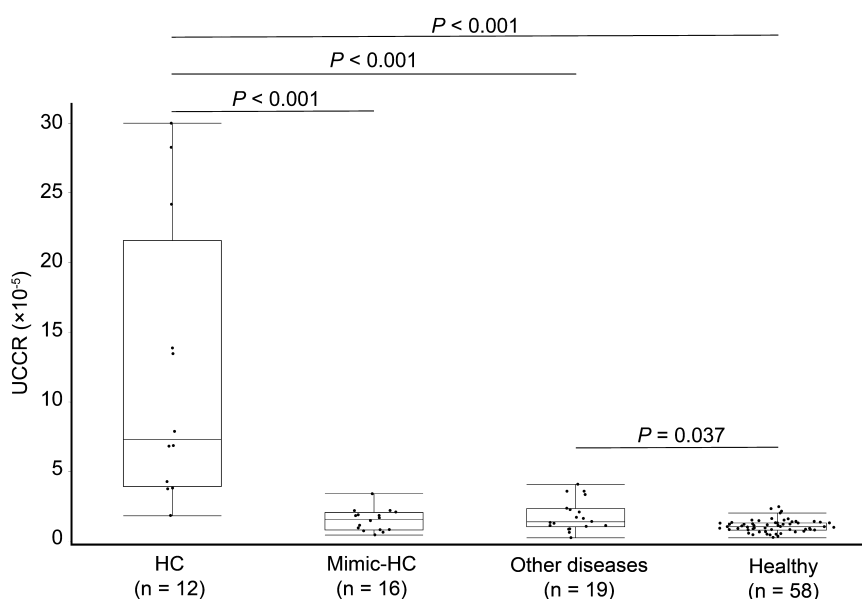
Urinary corticoid to creatinine ratios in clinical cases

UCCR was significantly higher in HC dogs than in mimic-HC dogs ($P<0.001$), dogs with other diseases ($P<0.001$), and healthy controls ($P<0.001$) (Fig. 1). It was also significantly higher in dogs with various diseases than in the healthy controls ($P=0.037$). The AUC for UCCR to differentiate HC dogs from mimic-HC dogs was 0.971, with a sensitivity of 91.7% and specificity of 100% when the cut-off value was set at 3.77×10^{-5}. When the cut-off value was set to 1.98×10^{-5}, which was derived from the UCCR value of the aforementioned healthy controls, the sensitivity and specificity were 91.7% and 75.0%, respectively.

When the UCCR of 16 paired urine samples collected at home and in the hospital were compared, the UCCR of urine collected in the hospital was significantly higher than that of urine collected at home (mean difference 3.30×10^{-5}, 95% confidence interval: 0.70–5.90, $P=0.001$) (Fig. 2).

Table 1. Comparison of clinical characteristics between groups

Variable	HC (n=12)	Mimic-HC (n=16)	*P* value
Median age, years (range)	12.8 (9.9–15.1)	11.4 (3.8–15.8)	0.44
Median weight, kg (range)	5.8 (3.7–32.7)	5.1 (1.7–16.7)	0.49
Female, number (%)	7 (58.3%)	11 (68.8%)	0.70
Polyuria/polydipsia, number (%)	8 (66.7%)	8 (50.0%)	0.46
Polyphagia, number (%)	5 (41.7%)	1 (6.3%)	0.057
Abdominal distension, number (%)	11 (91.7%)	4 (25.0%)	<0.001
Excessive panting, number (%)	3 (25.0%)	2 (12.5%)	0.62
Alopecia, number (%)	4 (33.3%)	6 (37.5%)	1.00
Thin skin, number (%)	6 (50.0%)	0 (0%)	0.003
Median plasma urea, mg/dl (range)	16.7 (7.6–48.9)	21.6 (7.7–40.3)	0.43
Median plasma creatinine, mg/dl (range)	0.5 (0.2–1.1)	0.8 (0.3–1.8)	0.085
Median plasma ALT, U/l (range)	223 (58–1,001)	72 (32–217)	<0.001
Median plasma ALP, U/l (range)	1,069 (116–1,226)	104 (50–1,226)	0.003
Median urine specific gravity (range)	1.017 (1.009–1.033)	1.024 (1.006–1.061)	0.114
Median urine cortisol, μg/dl (range)	11.5 (5.9–50.1)	3.4 (1.6–14.1)	0.002
Median urine creatinine, mg/dl (range)	48.1 (18.9–116.2)	100.2 (37.1–335.9)	0.016
Median UCCR (range)	7.38×10^{-5} (1.86–29.98)	1.59×10^{-5} (0.47–3.42)	<0.001

ALP, alkaline phosphatase; ALT, alanine aminotransferase; HC, hypercortisolism; UCCR, urinary corticoid to creatinine ratio.
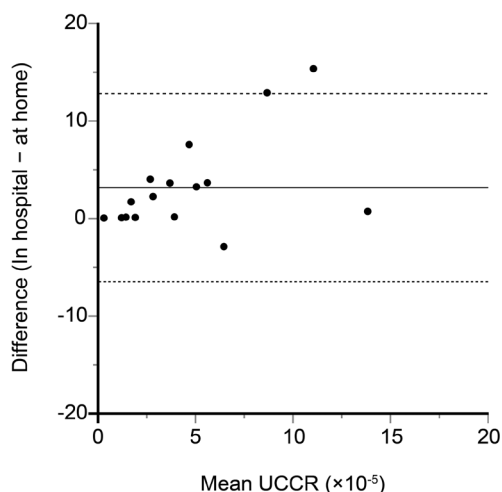

Fig. 1. Box and whisker plots of urinary corticoid to creatinine ratios (UCCR) in 12 dogs with hypercortisolism (HC), 16 dogs with mimic-HC, 19 dogs with other diseases, and 58 healthy dogs.

DISCUSSION

In the current study, the upper reference limit of UCCR measured by the IMMULITE 2000 XPi was determined in healthy dogs in Japan, and the diagnostic utility of this test was assessed in dogs with HC. The results of this study indicate that the measurement of UCCR using this method is a reliable endocrine test for the diagnosis of HC in dogs when urine is collected at home.

In the present study, the AUC of the UCCR for differentiating HC from mimic-HC in dogs was 0.971, which is comparable to or higher than that in previous studies. A previous study showed an AUC of 0.94, where a previous version of the CLEIA method, IMMULITE 1000, was used to measure urinary cortisol [28]. Another study that used enzyme-linked immunosorbent assay to determine urinary cortisol concentrations showed an AUC of 0.93 [14]. Our results support the clinical usefulness of UCCR for the diagnosis of canine HC, even when the latest CLEIA method is used.

In the present study, the upper reference limit for the UCCR derived from healthy controls was 1.98×10^{-5}, which is lower than that determined by a previous study (3.08×10^{-5}) using the IMMULITE 1000 [28]. Although the study included heavier dogs

Fig. 2. Bland–Altman difference plots of urinary corticoid to creatinine ratios (UCCR) collected at home and in hospital. The black line shows the mean difference. Dashed lines show the 95% limit of agreement.

(mean weight 22.1 kg) compared with our population (median weight 5.6 kg), other clinical findings, including age (mean 5.9 years versus median 4.5 years), were similar. Therefore, the difference in the analytical methods for urinary cortisol is a possible explanation for this difference. In fact, the British Small Animal Veterinary Association and the European Society of Veterinary Endocrinology indicated that the cortisol assay using IMMULITE 2000 showed lower values than previously measured due to changes in antibodies (https://www.bsava.com/News/ArticleID/2812). It should be noted that the cut-off values change according to the assay, laboratory, and even the kit lots.

The cut-off value derived from healthy controls in this study could effectively differentiate HC from mimic-HC with 91.7% sensitivity and 75.0% specificity, indicating that this value can be used in clinical situations. However, UCCR of one dog with HC in this study was within the reference range (1.86×10^{-5}). Because UCCR principally assesses cortisol production but not adrenocortical reserve or resistance to glucocorticoid feedback [1], UCCR within the reference range cannot exclude HC in dogs, and other endocrine tests such as ACTH stimulation test and LDDST should be performed in cases with clinical suspicion of HC. In fact, the results of ACTH stimulation test and LDDST were consistent with HC in the dog with UCCR within the reference range in this study. The specificity using the cut-off value derived from healthy controls in this study was higher than that reported in a previous study, where the specificity was 65% when the cut-off value was derived from similar controls [28]. This is probably due to the difference in the cases included in the mimic-HC groups. It is possible that clinically mild cases were included in the mimic-HC group in this study. Although there was no significant difference in UCCR between dogs with mimic-HC and healthy controls, a significant difference was detected between dogs with other diseases and healthy dogs. This indicates that the UCCR measurements were affected by disease type and severity. It is also noteworthy that the urine collection method clearly affects the UCCR values. The results of this study showed substantial differences between the UCCR values collected at home and in the hospital, which is in agreement with previous studies [6, 27]. In this study, the UCCR value in the hospital was up to 9.7 times higher than that at home, despite being measured on the same day. Because the diurnal variation of cortisol excretion in urine is unlikely in dogs [15, 28], the higher values in the hospital were probably due to stress. Thus, it is strongly recommended that UCCR measurements should be performed using free-catch urine at home to prevent false-positive results.

UCCR measurements have been reported to have high sensitivity for the diagnosis of HC in dogs (>90%), [8, 14, 24–26, 28] except for one study (75%) [16]. Although the specificity of UCCR for the diagnosis of HC in dogs was reported to be as low as 21% in previous studies [8, 16, 25], higher specificities of 77–81.9% were reported when free-catch urine was used [24, 28], as in our study. In this study, the specificity was 100% when the cut-off value was set at 3.77×10^{-5} while maintaining a high sensitivity of 91.7%. As described in previous studies, the UCCR can be used to confirm the diagnosis of HC in dogs [28].

There was a weak but significant negative correlation between UCCR and body weight in healthy controls in this study, which is consistent with the results of previous studies [5, 28]. This is probably because the smaller the dog, the smaller the muscle mass, resulting in a lower rate of creatinine excretion. In fact, there was also a significant negative correlation between the UCCR and serum creatinine concentration. Similarly, urine creatinine concentrations in dogs with HC was lower than those in dogs with mimic-HC. Dogs with HC have been reported to have smaller amount of muscle mass [22].

In this study, only 66.7% of dogs with HC exhibited polyuria/polydipsia, which is lower than that reported in previous studies, with 82–95% of the cases showing signs of polyuria and/or polydipsia [2, 3, 19, 21, 23]. However, the results of this study are similar to those of a study conducted in a primary care practice in Japan, with 41.9% of cases showing polyuria and polydipsia [20]. One possible reason for this difference is the difference in the populations included in the studies, especially the difference in dog breeds, which vary according to the country. It is possible that the clinical signs presented differ among dog breeds, although further studies are necessary to clarify the relationship between dog breeds and specific clinical signs. It should be noted that not all dogs with HC show polyuria and/or polydipsia.

This study has several limitations. The number of cases in each group was relatively small, emphasizing the necessity for future studies to validate the cut-off value established in this study. The other limitation was that not all dogs with mimic-HC were subjected to endocrine tests other than UCCR and none of the dogs had necropsy in this study; it is possible that the diagnosis of HC was overlooked in mimic-HC dogs, although the alternative clinical diagnosis was determined in each case.

In conclusion, UCCR determined by IMMULITE 2000 XPi is a useful diagnostic test for HC in dogs. Because the UCCR has high sensitivity and is an easy and non-invasive test, it can be used as a screening test for this disease. Furthermore, it can also be used as a confirmatory test when the cut-off value is increased. It should be noted that the urine collection method considerably affects the measurements.

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