The Veterinary Journal 252 (2019) 105343

Contents lists available at ScienceDirect

The Veterinary Journal

journal homepage: www.elsevier.com/locate/tvjl

Invited review

Diagnosis of spontaneous hyperadrenocorticism in dogs. Part 2: Adrenal function testing and differentiating tests

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ARTICLE INFO

Keywords:
Adrenals
Cortisol
Cushings
Endocrinology
Pituitary

ABSTRACT

Hyperadrenocorticism is a relatively common endocrine disorder in dogs that has been extensively described. However, its diagnosis remains challenging because there is no true reference standard test, and a myriad factors can affect the diagnostic performance of the commonly used adrenal function tests. Ultimately, the diagnosis is based on a combination of signalment, history and clinical findings, and a variety of diagnostic test results. The second part of this review aims to appraise available data on diagnostic performance of adrenal function tests in naturally occurring canine hyperadrenocorticism.

Introduction

A major issue when investigating canine hyperadrenocorticism (HAC) is that none of the currently available adrenal function tests are totally reliable, with frequent false-positive and false-negative results (Behrend et al., 2013). Many non-adrenal illnesses (NAIs) affect the results of adrenal function tests (Chastain et al., 1986; Kaplan et al., 1995). The ACVIM consensus statement on diagnosis of HAC (Behrend et al., 2013) suggested specific indications to maximize the prevalence of HAC in the population tested (pre-test probability) and consequently enhance the positive predictive value (PPV) of adrenal function tests. These include compatible history and physical examination findings, the presence of an adrenal mass or pituitary macrotumour, or poorly controlled diabetes mellitus despite high doses of insulin after exclusion of other causes. These have been reviewed previously (Bennaim et al., 2019). Adrenal function testing and imaging will be described in the following review.

Adrenal function tests

Urine corticoid-to-creatinine ratio

The diagnostic sensitivity of the urine corticoid-to-creatinine ratio (UCCR) varies between studies. Although generally agreed that it is high, with values from 92 to 100% (Stolp et al., 1983; Rijnberk et al., 1988; Feldman and Mack, 1992; Smiley and Peterson, 1993; Kaplan et al., 1995; Jensen et al., 1997; Zeugswetter et al., 2010), one study reported a sensitivity as low as 75% (Kaplan et al., 1995; Table 1), which may be related to different selection criteria or small sample population size.

Specificity of the UCCR for diagnosing HAC ranges from 21 to 100% (Stolp et al., 1983; Rijnberk et al., 1988; Feldman and Mack, 1992; Smiley and Peterson, 1993; Kaplan et al., 1995; Jensen et al., 1997; Zeugswetter et al., 2010). Several pre-analytical (sample collection and control population) and analytical factors (assay methodology) influence diagnostic test specificity. The UCCR in urine collected after a physical examination and blood sampling was not significantly different to that in urine collected after six days at home (Galeandro et al., 2014). However, hospitalisation of healthy dogs for 1.5 days significantly increased the UCCR compared to values in urine samples collected at home (van Vonderen et al., 1998). The lowest specificities of the UCCR (from 21 to 24%), were reported in hospitalised dogs (Smiley and Peterson, 1993; Kaplan et al., 1995) or the environmental conditions were not specified (Feldman and Mack, 1992). The method of urine collection for the UCCR has never been specifically evaluated. Specificity may have been further underestimated if cystocentesis or catheterisation were used for urine collection (Smiley and Peterson, 1993; Kaplan et al., 1995; Jensen et al., 1997).

Differences between control groups can affect diagnostic test specificity (Table 1). The highest specificity (from >95 to 100%) was documented using control groups comprised of healthy dogs or dogs with NAI in which HAC was not clinically suspected (Smiley and Peterson, 1993; Kaplan et al., 1995; Jensen et al., 1997). Such control populations are unlikely to reflect the type of dogs tested in clinical practice. A lower specificity of 85% was reported in dogs in which HAC was considered as a possible differential (Jensen et al., 1997). In that study some dogs were classified as controls based on

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http://dx.doi.org/10.1016/j.tvjl.2019.105343
1090-0233/

Table 1
Summary of the studies evaluating the performance of the urine corticoid: creatinine ratio for diagnosis of canine spontaneous hyperadrenocorticism.

	Stolp et al. (1983)	Rijnberk et al. (1988)	Feldman and Mack (1992)	Smiley and Peterson (1993)	Kaplan et al. (1995)	Jensen et al. (1997)	Zeugswetter et al. (2010)
Method of urine collection and environmental conditions	Free catch	Free catch	Free catch	Various methods	Various methods	Various methods	Free catch
	Environmental conditions not specified	At home	Environmental conditions not specified	During hospitalisation for control dogs	During hospitalisation for control dogs	At home or upon arrival at hospital	At home
Number of HAC dogs	27	93	40	25	20	18	66
Number of control dogs	28 (healthy)	57 (NAI mimicking HAC)	20 (healthy)	31 (healthy)	21 (healthy)	20 (NAI mimicking HAC)	87 (NAI mimicking HAC)
			23 (polyuria/polydipsia but HAC excluded)	21 (mild NAI mimicking HAC)	59 (various NAI, HAC not suspected)		
				28 (various moderate-to-severe NAI, HAC not suspected)			
Assay	RIA	RIA	RIA	RIA	RIA	ELISA	CLIA
Sensitivity	100%	99%	100%	92%	75%	100%	92.4%
Specificity	100%	77%	100% using healthy dogs	97% using healthy dogs	>95% using healthy dogs	85%	81.9%
			22% using the group of dogs with polyuria/ polydipsia	95% using the group of dogs with mild NAI mimicking HAC 21% using dogs with moderate-to-severe NAI	24% using dogs with NAI		

CLIA, chemiluminescent immunoassay; ELISA, enzyme-linked immunosorbent assay; HAC, hyperadrenocorticism; NAI, non-adrenal illness; RIA, radioimmunoassay.

a negative adrenocorticotropic hormone (ACTH) stimulation test; however, it is inappropriate to use this test to exclude HAC because of the high proportion of false negative results (Meijer et al., 1979; Feldman, 1981, 1983a; Peterson et al., 1982a; Reusch and Feldman, 1991; Kaplan et al., 1995; Van Liew et al., 1997), leading to the possibility that some dogs with HAC might have been included in the control group.

Analytical bias is also important. Five different assays were used in the studies evaluating the diagnostic performance of the UCCR (Stolp et al., 1983; Rijnberk et al., 1988; Feldman and Mack, 1992; Smiley and Peterson, 1993; Kaplan et al., 1995; Jensen et al., 1997; Zeugswetter et al., 2010). Rijnberk et al. (1988) used an in-house radioimmunoassay (RIA) only available in the Netherlands that uses anti-cortisol antibodies with little cross-reactivity to cortisol metabolites. Other assays may have greater cross reactivity and should be interpreted with reference intervals or decision thresholds that are method, assay, and laboratory-specific (Zeugswetter et al., 2010, 2013). If such specific decision thresholds are used and under ideal pre-analytical conditions (free-catch urine samples collected at home and control population of dogs in which HAC was initially suspected but ultimately ruled out), the reported specificities of 77% (Rijnberk et al., 1988) and 81.9% (Zeugswetter et al., 2010) become more closely aligned.

Other factors that may affect the performance of the UCCR include the recent administration of glucocorticoids or progestagens. Some exogenous glucocorticoids (e.g., prednisolone) may cross-react in cortisol assays and both exogenous glucocorticoids and progestagens may, in the long-term, suppress the hypothalamic-pituitary-adrenal axis and consequently decrease the UCCR (Selman et al., 1997). Phenobarbital therapy does not affect the UCCR (Foster et al., 2000).

Low-dose dexamethasone suppression test

The diagnosis of HAC with the low-dose dexamethasone suppression test (LDDST) relies on the demonstration of decreased response of the hypothalamic-pituitary-adrenal axis to negative glucocorticoid feedback (Behrend, 2015). For this test, dexamethasone (0.010–0.015 mg/kg) is administered intravenously and samples collected for serum cortisol measurement before, 3–4 h, and 8 h later (Mack and Feldman, 1990; Behrend et al., 2013). In healthy dogs, dexamethasone results in rapid and prolonged suppression of cortisol secretion (Meijer et al., 1978). A cortisol concentration above the laboratory cut-off 8 h after dexamethasone administration has historically been considered abnormal, and consistent with HAC (Rijnberk et al., 1988; Reusch and Feldman, 1991; Kaplan et al., 1995; Van Liew et al., 1997).

The diagnostic sensitivity of the LDDST varies between studies, primarily because of different criteria used to confirm a diagnosis. In two studies reporting a high sensitivity of 99% (Reusch and Feldman, 1991) and 100% (Kaplan et al., 1995), some dogs were included in the HAC group based at least in part upon a positive LDDST, which is inappropriate and likely to overestimate sensitivity. In another study, reporting a sensitivity of 96%, post-mortem examination was an inclusion criteria (Van Liew et al., 1997), and therefore likely included more severely affected cases, again overestimating sensitivity. However, sensitivity remained between 85 and 97% in the other studies in which HAC was diagnosed based on a combination of compatible clinical and clinicopathological findings, response to treatment and post-mortem examination (when available), with or without supportive adrenal function test results (ACTH stimulation test or UCCR; Feldman, 1983a; Rijnberk et al., 1988; Bennaim et al., 2018), suggesting the LDDST is truly a sensitive test for diagnosing this disease.

The reported specificity of the LDDST ranges from 44 to 95% (Table 2; Chastain et al., 1986; Rijnberk et al., 1988; Van Liew et al., 1997; Bennaim et al., 2018). The discrepant results between studies are, in part, explained by the use of different control groups. The lowest reported specificities (44 and 48.5%) were obtained using dogs with various NAIs in which HAC was not clinically suspected (Chastain et al., 1986; Kaplan et al., 1995), while the highest

Table 2
Summary of the studies evaluating the performance of the low-dose dexamethasone suppression test for diagnosis of canine spontaneous hyperadrenocorticism.

	Feldman (1983a)	Rijnberk et al. (1988)	Chastain et al. (1986)	Reusch and Feldman (1991)	Kaplan et al. (1995)	Van Liew et al. (1997)	Bennaim et al. (2018)
Assay	RIA	RIA	RIA	RIA	RIA	Not specified	CLIA
Number of HAC dogs	64	129	None	85	20	28 (post-mortem)	59
Control group	None	37 (NAI mimicking HAC)	33 (various NAI)	None	21 healthy dogs	10 (NAI mimicking HAC, which was excluded with post-mortem)	64 (NAI mimicking HAC)
					59 dogs (various NAI, HAC not suspected)		
Sensitivity	92%	85%	Not applicable	99%	100%	96%	97%
Specificity	Not applicable	73%	48.5%	Not applicable	>95% using healthy dogs	70%	67%
					44% using dogs with various NAI		

CLIA, chemiluminescent immunoassay; HAC, hyperadrenocorticism; NAI, non-adrenal illness; RIA, radioimmunoassay.

reported specificity was obtained using a group of healthy dogs (Kaplan et al., 1995). Van Liew et al. (1997) reported a specificity of 70% using a population of dogs with NAIs mimicking HAC and in which post-mortem examination was performed. Whilst a more appropriate control group, the requirement for necropsy may have resulted in the inclusion of more severely ill dogs, potentially resulting in underestimation of specificity. Only two studies evaluated the specificity of the LDDST in a group of dogs in which HAC was initially clinically suspected but later excluded without requirement for a necropsy (Rijnberk et al., 1988; Bennaim et al., 2018). Both studies reported a moderate specificity of approximately 70%.

Most of the above studies used a cut-off of 40 nmol/L for the 8 h cortisol concentration (Feldman, 1983a; Chastain et al., 1986; Rijnberk et al., 1988; Reusch and Feldman, 1991; Kaplan et al., 1995; Van Liew et al., 1997) and one study used an additional cut-off of 52 nmol/L for the 3-h cortisol concentration (Chastain et al., 1986), all using RIAs. Other non-isotopic methods for cortisol measurement have now become more popular. These include chemiluminescent immunoassays (Reimers et al., 1996; Singh et al., 1997), enzyme-linked immunosorbent assays (Ginel et al., 1998) and fluorometric immunoassays (Jericó et al., 2002). Many laboratories using these methods maintain the cut-off of 40 nmol/L extrapolated from RIA studies. However, measured cortisol concentrations differ both between methods and laboratories. Additionally, cut-offs may have previously been derived from studies with various shortcomings (Behrend et al., 2013). Cut-offs values would ideally be evaluated per method and per laboratory, although this is rarely the case in practice.

Several LDDST patterns have been described, including lack of suppression, partial suppression, escape, inverse, and complete suppression (Fig. 1). Some of these patterns are used to differentiate pituitary-dependent HAC (PDH) from a functional adrenocortical tumour (AT). As previously described, most studies evaluating the diagnostic performance of the LDDST for HAC considered the lack of suppression, partial suppression and escape patterns as positive results and complete suppression patterns as negative results. Although historically considered a negative LDDST result, the inverse pattern has recently been suggested as supportive of PDH in one report (Mueller et al., 2006).

A recent study evaluated the PPV of individual LDDST patterns for diagnosing HAC in a population of dogs in which the disease was clinically suspected and subsequently confirmed or ruled out (Bennaim et al., 2018). Lack of suppression was the most common pattern in dogs with HAC (approximately 50% of cases) and was associated with the highest PPV (94%) in this population. The PPV of the partial suppression pattern was not significantly different compared to the LDDST overall (73% each). By contrast escape and inverse patterns were associated with a PPV (37%) significantly lower than the PPV of the LDDST overall. A complete suppression pattern was associated with a high negative predictive value (96%). Based on these results, suggested interpretation of the LDDST is provided in Fig. 2.

Recent administration of glucocorticoids or progestagens may suppress the hypothalamic-pituitary-adrenal axis and consequently affect the LDDST (Selman et al., 1997). Available evidence suggests no effect of phenobarbital therapy on the LDDST (Chauvet et al., 1995; Foster et al., 2000; Müller et al., 2000). However, considering occasional reports of inadequate suppression of cortisol secretion during a LDDST (Chauvet et al., 1995; Foster et al., 2000) and the difficulty in distinguishing some clinical signs and clinicopathological abnormalities supportive of HAC from the side effects of phenobarbital therapy, the ACVIM consensus statement advises to ideally switch to another anticonvulsant prior to testing or, if not possible, interpret results cautiously (Behrend et al., 2013). It is generally accepted that stressful procedures should be avoided during a LDDST (May et al., 2004).

Adrenocorticotropic hormone stimulation test

The ACTH stimulation test assesses adrenocortical reserve. Serum cortisol concentrations are measured before and at 60–90 min after administration of a pharmacological dose of synthetic ACTH (also known as tetracosactide or cosyntropin; Behrend et al., 2013). A recent study has suggested that the measurement of basal cortisol concentration is redundant in the investigation of PDH because of the good discriminatory ability of the ACTH-stimulated cortisol concentration (Nivy et al., 2018). However, that study did not include dogs with functional ATs. A small difference between baseline and ACTH-stimulated cortisol concentration constitutes one of the criteria to diagnose the subset of functional ATs secreting hormones other than cortisol (see below). Additionally, the difference between baseline and ACTH-stimulated cortisol concentration is also used to identify dogs with iatrogenic HAC. Therefore, the authors of this review would still recommend measuring baseline cortisol concentration when performing an ACTH stimulation test.

Synthetic ACTH is commercially available in liquid, lyophilised or depot forms, although availability varies both geographically and temporally. Compounded forms of naturally occurring ACTH, often of unknown type of source, are also occasionally used. No significant differences were found between cortisol response following the use of either liquid (250 μg/dog IV) or lyophilised (250 μg/dog IM) solutions of tetracosactide in 18 healthy dogs

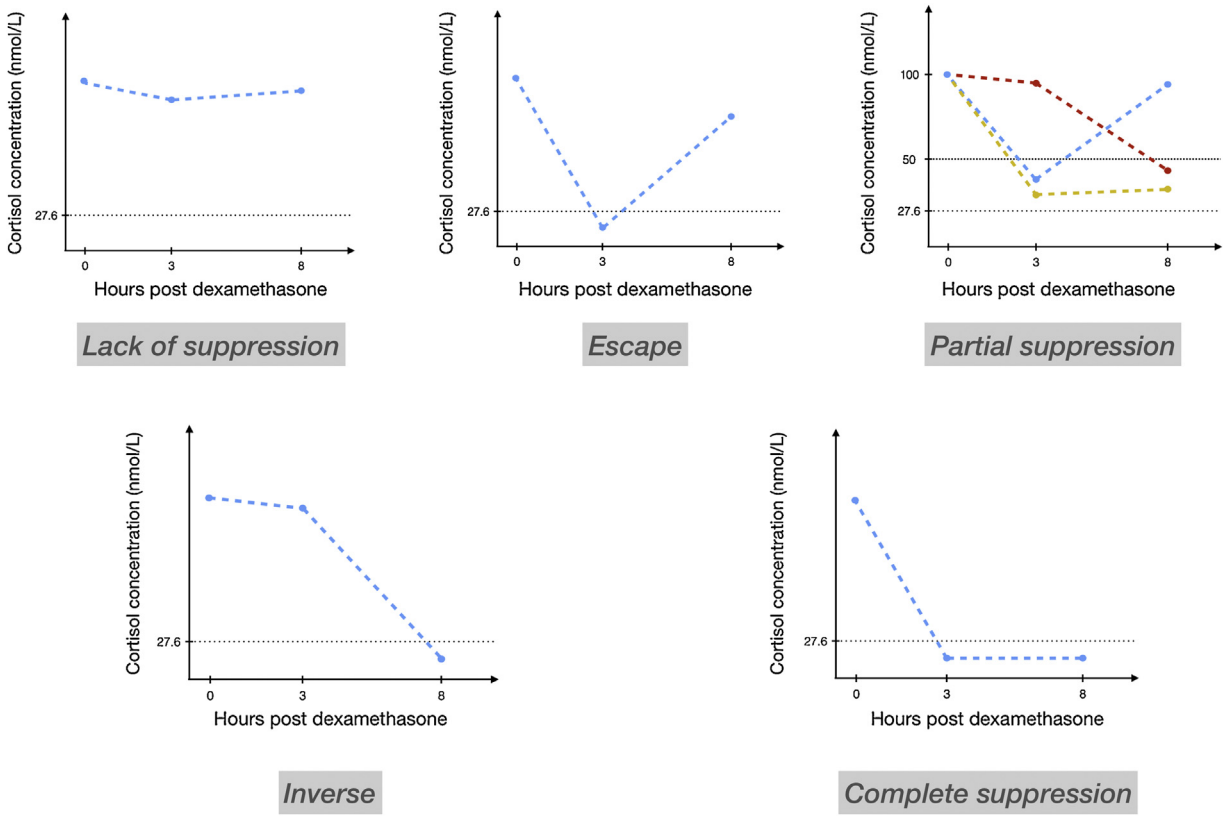

Fig. 1. Illustration of different low-dose dexamethasone suppression test patterns including the lack of suppression (3- or 4-h, and 8-h cortisol concentration above the laboratory cut-off and both >50% basal cortisol concentration), partial suppression (3- or 4-h, and 8-h cortisol concentration above the laboratory cut-off but either <50% basal cortisol concentration), escape (3- or 4-h cortisol concentration below and 8-h cortisol concentration above the laboratory cut-off), inverse (3- or 4-h cortisol concentration above and 8-h cortisol concentration below the laboratory cut-off), and complete suppression (3- or 4-h, and 8-h cortisol concentrations both below the laboratory cut-off). A laboratory cut-off of 27.6 nmol/L was used.

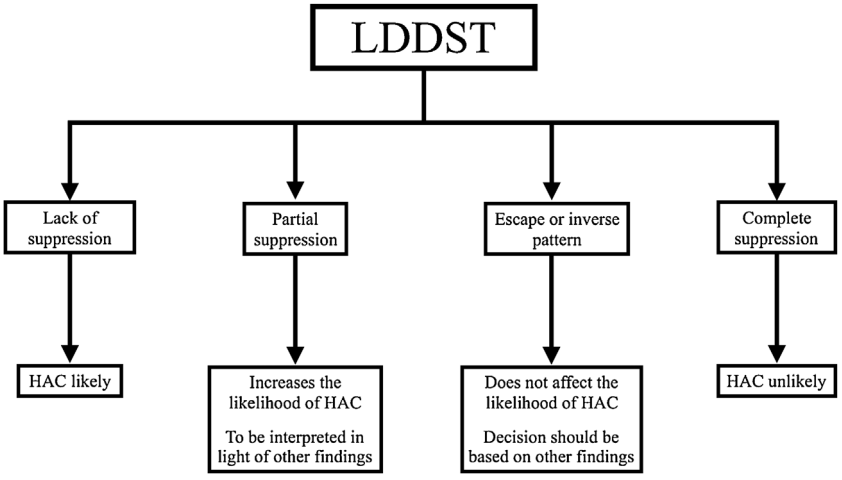

Fig. 2. Flow-chart with interpretation of the low-dose dexamethasone suppression test (LDDST) when performed in dogs in which there is a clinical suspicion of hyperadrenocorticism (HAC) with or without supportive laboratory and imaging findings.

(Cohen and Feldman, 2012). In another study, cortisol concentrations at 60 min after injection of tetracosactide (5 μg/kg IV) or four compounded ACTH preparations (2.2 units/kg IM) were not significantly different (Kemppainen et al., 2005). However, these studies only included healthy dogs and the results cannot be directly extrapolated to dogs in which HAC is clinically suspected. One pilot study showed that the use of a compounded form of ACTH (2.2 units/kg, IM) instead of tetracosactide (250 μg/dog IM) in dogs with clinical signs of HAC or hypoadrenocorticism resulted in lower post-ACTH cortisol concentrations that could potentially lead to different clinical interpretation (Hill et al., 2004). Additionally, compounded preparations may contain variable concentrations of ACTH; in one study immunoreactive ACTH content in four different preparations ranged from 11 to 925 μg/mL, despite all being labelled as containing 40 IU/mL (Kemppainen et al., 2005).

Doses of 5 μg/kg or 250 μg/dog of tetracosactide can be used for this test (Frank et al., 2000). Considering that the bioactivity of

tetracosactide is retained when frozen at −20 °C for up to 6 months (Frank and Oliver, 1998), the use of 5 μg/kg allows multiple uses of a single vial. In dogs in which HAC was suspected, an ACTH stimulation test using a lower dose of tetracosactide (1 μg/kg IV) when compared to using the higher dose (5 μg/kg IV) resulted in a different clinical interpretation in approximately 25% of cases, with HAC more likely to be diagnosed when using the higher dose (Aldridge et al., 2016). Therefore, the lower dose should not be used in dogs suspected of HAC.

As part of the ACTH stimulation test, tetracosactide can be administered either IM or IV without affecting results (Behrend et al., 2006). Intramuscular administration appears to be painful in some dogs.

Post-ACTH serum cortisol concentration should be assessed on a sample collected 60 to 90 min after tetracosactide administration (Hansen et al., 1994; Kerl et al., 1999; Frank et al., 2000; Kemppainen et al., 2005; Behrend et al., 2006). If compounded forms of ACTH are used, sample collection should occur 60 min after injection. As mentioned earlier, serum cortisol concentrations were similar at 60 min after injection of tetracosactide or the various compounded forms of ACTH (Kemppainen et al., 2005). However, ACTH-stimulated cortisol concentrations varied considerably between preparations at later times. In one study in which a depot formulation was used peak cortisol secretion occurred between two and four hours in all 15 dogs in which HAC was suspected, and a sampling time of 180 min is recommended for this formulation (Sieber-Ruckstuhl et al., 2015).

No significant difference was observed between post-ACTH serum cortisol concentrations (using ACTH gel at standard dose) when the test was performed in healthy dogs either at home, or after four or 12 h hospitalised in a cage, suggesting that the testing environment minimally affects results (Vial et al., 1979).

The reported sensitivity of the ACTH stimulation test ranges from 0 to 95% with different populations contributing to the varied results (Tables 3 and 4; Meijer et al., 1979; Feldman, 1981, 1983a; Peterson et al., 1982a; Reusch and Feldman, 1991; Kaplan et al., 1995; Van Liew et al., 1997; Norman et al., 1999; Nivy et al., 2018). The frequency of false negative test results appears higher in dogs with functional AT as compared to dogs with PDH. The sensitivity of the ACTH stimulation test reported for dogs with functional AT ranges from 0 to 63% (Meijer et al., 1979; Feldman, 1981, 1983a; Peterson et al., 1982a; Reusch and Feldman, 1991; Norman et al., 1999), while the sensitivity reported for dogs with PDH ranges from 80 to 92% (Feldman, 1983a; Reusch and Feldman, 1991; Kaplan et al., 1995; Nivy et al., 2018). Some studies may have overestimated sensitivity due to the inclusion of more advanced or severely affected cases, especially if a post-mortem examination was required for inclusion (Van Liew et al., 1997). Finally, in three other studies, one reporting sensitivities of 63% for functional AT and 88% for PDH (Reusch and Feldman, 1991) and two, comprising only dogs with PDH, reporting sensitivities of 80% (Kaplan et al., 1995) and 92% (Nivy et al., 2018), some dogs appear to have been included in the HAC group based, in part, upon a positive ACTH stimulation test, which is not appropriate when evaluating the diagnostic properties of the same said test.

The low sensitivity of the ACTH stimulation test for functional ATs occurs if an exaggerated response to ACTH administration were expected. Some functional ATs may predominantly secrete hormones other than cortisol and a subnormal cortisol response to synthetic ACTH may be observed. Therefore, dogs exhibiting clinical signs of HAC without recent steroid administration, but with absent to weak stimulation of cortisol secretion could be interpreted as indicative of an AT (Norman et al., 1999; Syme et al., 2001; Shiel et al., 2010; Bennaim et al., 2018). Considering these changes as a positive result would increase sensitivity of the test for functional AT. In two studies, the proportion of dogs with subnormal cortisol response represented 37.5% of cases with functional AT (Norman et al., 1999; Bennaim et al., 2018). However, this proportion was lower in other studies (0-11.1%; Meijer et al., 1979; Feldman, 1981; Peterson et al., 1982a; Feldman, 1983a; Reusch and Feldman, 1991).

The reported specificity of the ACTH stimulation test ranges from 59 to 95% (Tables 3 and 4; Chastain et al., 1986; Kaplan et al., 1995; Van Liew et al., 1997; Behrend et al., 2005; Prittie et al., 2005; Monroe et al., 2012; Nivy et al., 2018). The evaluation of specificity is also limited by variation in the control populations used. Most used either healthy dogs (Kaplan et al., 1995) or dogs with NAIs in which HAC was not suspected (Chastain et al., 1986; Kaplan et al., 1995; Van Liew et al., 1997; Behrend et al., 2005; Prittie et al., 2005; Monroe et al., 2012), which do not reflect the use of the test in a clinical setting. In another study with a high specificity of 91%, necropsy was necessary for inclusion in the NAI group (Van Liew et al., 1997). In the remaining studies evaluating the test in dogs with NAI mimicking HAC, without requirement for a necropsy, the

Table 3
Summary of studies evaluating the diagnostic performances of the adrenocorticotropic hormone stimulation test.

	Meijer et al. (1979)	Feldman (1981)	Peterson et al. (1982a)	Feldman (1983a)	Chastain et al. (1986)	Reusch and Feldman (1991)
Assay	RIA	Fluorometric method ($n = 5$) and competitive binding method ($n = 1$)	RIA	RIA	RIA	RIA
HAC group	Nine dogs with functional AT	Six dogs with functional AT	22 dogs with functional AT	64 dogs ($n = 26$ PDH; $n = 7$ functional AT; $n = 31$ not specified)	None	85 dogs ($n = 44$ PDH; $n = 41$ functional AT)
Control group	None	None	None	None	33 dogs with various NAI (HAC not suspected)	None
Sensitivity	33% (one dog had subnormal cortisol response, this result was considered negative)	50% (some dogs had contradictory results when the test was repeated)	59%	83% overall 88% for dogs with PDH 57% for dogs with functional AT	Not applicable	88% in dogs with PDH 63% in dogs with functional AT
Specificity	Not applicable	Not applicable	Not applicable	Not applicable	64%	Not applicable

AT, adrenocortical tumour; HAC, hyperadrenocorticism; NAI, non-adrenal illness; PDH, pituitary-dependent hyperadrenocorticism; RIA, radioimmunoassay.

Table 4
Summary of studies evaluating the diagnostic performances of the adrenocorticotropic hormone stimulation test.

	Kaplan et al. (1995)	Van Liew et al. (1997)	Norman et al. (1999)	Behrend et al. (2005)	Prittie et al. (2005)	Monroe et al. (2012)	Nivy et al. (2018)
Assay	RIA	Not specified	RIA	RIA	RIA	RIA	CLIA
HAC group	20 dogs with PDH	22 dogs (cause unspecified) diagnosed based on post-mortem	8 dogs with functional AT	None	None	32 dogs (n = 28 PDH; n = 3 functional AT; n = 1 AT and pituitary carcinoma)	36 dogs with PDH
Control group	21 healthy dogs 59 dogs with various NAI in which HAC was not suspected	10 dogs with NAI mimicking HAC and in which HAC was excluded with post-mortem	None	35 dogs with NAI (non-adrenal neoplasia) in which HAC was not suspected	20 with various NAI in which HAC was not suspected	29 dogs with NAI mimicking HAC	18 dogs with NAI mimicking HAC
Sensitivity	80%	95%	0 if only post-ACTH cortisol above the cut-off considered positive 37.5% if subnormal cortisol response considered positive	Not applicable	Not applicable	84%	92% using conventional cut-off value 86% using optimal cut-off value
Specificity	>95% using healthy dogs 86% using dogs with NAI	91%	Not applicable	91%	90%	59%	61% using conventional cut-off value 94% using optimal cut-off value

ACTH, adrenocorticotropic hormone; AT, adrenocortical tumour; CLIA, chemiluminescent immunoassay; HAC, hyperadrenocorticism; NAI, non-adrenal illness; PDH, pituitary-dependent hyperadrenocorticism; RIA, radioimmunoassay.

reported specificities were 59 (Monroe et al., 2012) and 61% (Nivy et al., 2018), which challenges the widespread belief that this test is highly specific. In one of these studies (Nivy et al., 2018), an post-ACTH serum cortisol concentration cut-off value (683 nmol/L) higher than that conventionally used by most laboratories (550–600 nmol/L) was reported as optimal and was associated with a higher specificity (94%) with an acceptable sensitivity (86%). Although different post-ACTH serum cortisol concentration cut-offs values may be associated with overall better diagnostic accuracy, these should ideally be established in an appropriate population, specific to the method, assay and laboratory used.

The ACTH stimulation test is also the reference standard for the diagnosis of iatrogenic HAC, in which a decreased cortisol response is expected (Zenoble and Kemppainen, 1987; Eichenbaum et al., 1988; Moriello et al., 1988; Behrend and Kemppainen, 1997).

A suggested interpretation of the ACTH stimulation test is provided in Fig. 3.

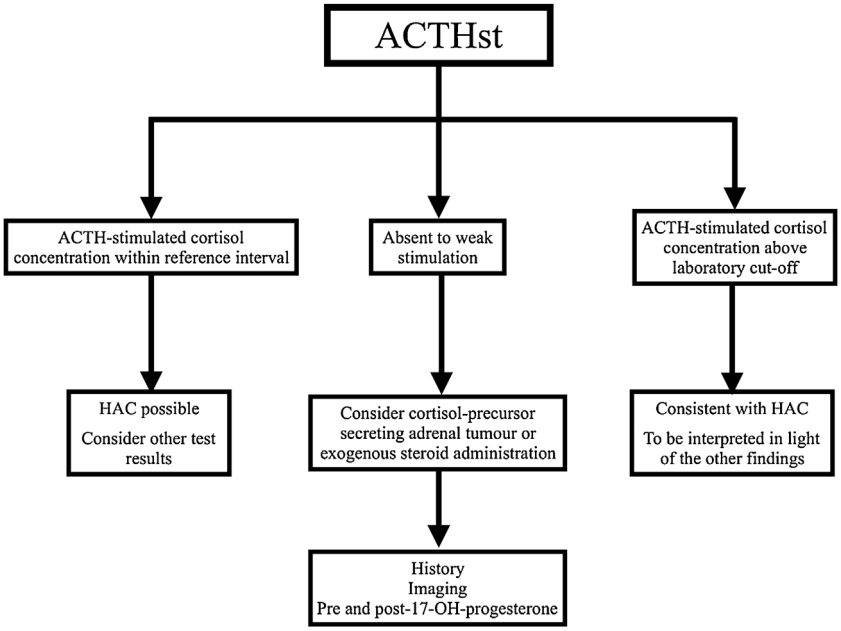

Fig. 3. Flow-chart with interpretation of the adrenocorticotropic hormone stimulation test (ACTHst) when performed in dogs in which there is a clinical suspicion of hyperadrenocorticism (HAC) with or without supportive laboratory and imaging findings.

Combined urine corticoid-to-creatinine ratio and low-dose dexamethasone suppression test

A combined UCCR and LDDST has been evaluated as a screening test for HAC (Kooistra and Galac, 2012). Urine is collected followed by the administration of an oral dose of 0.01 mg/kg of dexamethasone with a second urine sample collected 8-h later. The 8-h UCCR of seven healthy dogs was lower than 1.0×10^{-6} in one study using an assay with little cross-reactivity with cortisol metabolites (Vaessen et al., 2004). This test requires further evaluation, notably in dogs in which HAC is clinically suspected.

Negative adrenal function test results in dogs with hyperadrenocorticism

The ACVIM consensus statement for the diagnosis of HAC defines occult (or atypical) HAC as a syndrome in which clinical and clinicopathological findings are consistent with HAC but in which the LDDST, UCCR and ACTH stimulation test results fall into accepted reference intervals (Behrend et al., 2013). This may not be an appropriate term because occult disease refers to the presence of a disease in the absence of clinical signs, and these cases may not be atypical in their clinical presentation. Some dogs fulfilling the criteria for so-called occult or atypical HAC belong to the subset of functional ATs associated with weak to absent cortisol stimulation following ACTH administration. Indeed, low basal, 3-h and 8-h cortisol concentrations during the LDDST is occasionally observed in these cases (Norman et al., 1999; Syme et al., 2001).

Although cases without AT that fulfil the definition for occult or atypical HAC have been recognised, they may not represent a distinct entity. The negative results may simply reflect the lack of perfect test sensitivity as observed with any diagnostic test. One study reported that some dogs with 'occult' HAC without AT secrete excessive amounts of cortisol compared to healthy dogs, albeit lower than that in dogs with PDH and positive adrenal function tests (Frank et al., 2015). These findings, along with similar clinical signs compared to dogs with typical HAC suggest that cortisol excess may be part of the pathophysiology of 'occult' HAC and these dogs may have greater hypothalamic-pituitary cortisol sensitivity, as has been reported in humans (Huizenga et al., 1998). However, the amount of cortisol secreted was not compared to dogs with other illnesses, in which typical signs of HAC are not observed. Additionally, the criteria used to diagnose 'occult' HAC in this study are controversial and it is possible that some of these dogs had NAI.

Other potential explanations to explain the negative results of adrenal function testing in these dogs (Behrend et al., 2013) include that (1) the cut-off values used for adrenal function testing may be too high, resulting in imperfect sensitivity or (2) some dogs fulfilling the criteria for 'occult' HAC may have other and rarer forms of HAC (e.g., food-dependent HAC).

Adrenal function tests for differentiation

Following diagnosis of HAC, it is important to differentiate PDH from a functional AT, as treatment and prognosis may significantly differ. Indeed, while medical therapy is possible for functional ATs (Feldman and Mack, 1992; Kintzer and Peterson, 1991; Benchekroun et al., 2008; Helm et al., 2011; Arenas et al., 2014), adrenalectomy is ideally performed because signs may develop as a result of local tumour effects and as surgery may be curative (Behrend, 2015). Dogs with PDH may enjoy clinical remission and prolonged survival with medical therapy alone (Rijnberk and Belshaw, 1988; Kintzer and Peterson, 1991; Neiger et al., 2002; Ruckstuhl et al., 2002; Braddock et al., 2003; Alenza et al., 2006; Clemente et al., 2007; Vaughan et al., 2008; Galac et al., 2009), although hypophysectomy (Meij et al., 1998; Hanson et al., 2005; van Rijn et al., 2016) or radiation therapy (Dow et al., 1990; Théon and Feldman, 1998; Bley et al., 2005; de Fornel et al., 2007) are performed either routinely or in selected cases in some institutions.

Low-dose dexamethasone suppression test

A suppression greater than 50% of cortisol secretion 3- or 8-h post-dexamethasone administration or a three-hour post-dexamethasone cortisol concentration below the laboratory cut-off confirms the presence of PDH with high certainty in almost all cases. Indeed, only two cases of AT with such a pattern have been identified (Norman et al., 1999). However, these cases had low basal cortisol concentrations (33 and 30 nmol/L, respectively) approaching the cut-off value for the test. One study reported increasing cortisol concentrations (defined as an increase >50% in cortisol concentration between any time without suppression) during a LDDST in six of 59 dogs with HAC, all diagnosed as PDH (Bennaim et al., 2018). It could be hypothesized that a marked increase in cortisol concentration suggests ACTH secretion and, therefore, would only be found in dogs with PDH. However, dogs with functional AT secrete cortisol in an episodic and random manner and dramatic increase in cortisol concentrations may also occur despite absence of ACTH secretion although this is poorly described (Behrend, 2015). Therefore, additional studies are required prior to considering that such an increase in cortisol concentrations supports PDH.

Approximately half of dogs with HAC are diagnosed with PDH based solely upon the results of the LDDST. Indeed, a partial suppression, escape or inverse pattern (Fig. 1) will be identified in approximately 60% of dogs with PDH (Feldman et al., 1996; Bennaim et al., 2018). It is unclear why a cut-off of 50% is used to differentiate PDH from functional AT in earlier studies (Mack and Feldman, 1990; Feldman et al., 1996), although lesser degrees of suppression have not been evaluated. Lack of suppression on a LDDST can be identified in both PDH and functional AT. In fact, in dogs with HAC in which the frequency of PDH and functional AT are 80 to 85% and 15 to 20%, respectively (Feldman, 1983a; Alenza and Melian, 2015), the approximate equal chance of PDH versus functional AT becomes more likely with a lack of suppression pattern.

High-dose dexamethasone suppression test

The high-dose dexamethasone suppression test (HDDST) is performed and interpreted similarly to the LDDST but using a dose tenfold higher (0.1 mg/kg dexamethasone, IV; Feldman et al., 1996). Although lack of suppression is found in all dogs with functional AT, it also occurs in approximately 25% of dogs with PDH. Only 14% of all dogs with PDH that do not suppress on the LDDST suppress on the HDDST (Feldman et al., 1996). As a result, it will provide a definitive diagnosis in only approximately one third of dogs that display a lack of suppression with the LDDST. Other methods allowing differentiation between PDH and functional AT in a higher proportion of cases are more commonly used.

The UCCR can also be evaluated during a HDDST (Galac et al., 1997). Urine is collected prior to and after administration of dexamethasone (0.1 mg/kg PO) at eight-hour intervals for a total of three doses. A suppression of the UCCR greater than 50% is considered consistent with PDH. In this study, 80 of 111 (72%) dogs with PDH but none of the 49 dogs with functional AT showed greater than 50% suppression with this test.

Endogenous adrenocorticotropic hormone concentration

Pituitary-dependent HAC is caused by excessive pituitary secretion of ACTH whilst the excessive secretion of cortisol by

functional ATs results in suppression of ACTH secretion. Therefore, plasma endogenous ACTH (eACTH) concentration is expected to be high in dogs with PDH but low in dogs with functional ATs.

Measurement of eACTH concentration is not without difficulty. As canine ACTH is extremely labile, failure to properly collect and store plasma samples will result in erroneously low results. Plasma proteases rapidly degrade eACTH and to prevent this, blood is collected into refrigerated silicon-coated glass or plastic EDTA tubes, centrifuged within 15 min of collection, the plasma transferred to plastic tubes and immediately frozen, then shipped frozen (typically overnight, packed in dry ice) to the laboratory (Hegstad et al., 1990; Scott-Moncrieff et al., 2003; Behrend et al., 2013). The addition of the protease inhibitor aprotinin prevents ACTH degradation (Hegstad et al., 1990) but at standard concentrations (i.e., 50 IU/mL) results in a significant artefactual decrease in eACTH concentration if measured with a chemiluminescent, but not immunoradiometric, assay (Scott-Moncrieff et al., 2003).

Several eACTH assays have been evaluated in dogs. Older single-site assays have been replaced by more modern and accurate sandwich techniques (Feldman, 1983b; Gosling, 1990; Reusch and Feldman, 1991; Selby, 1999; Gould et al., 2001; Scott-Moncrieff et al., 2003; Rodríguez Piñeiro et al., 2009; Zeugswetter et al., 2013). The ability to differentiate PDH from functional AT based on eACTH concentration appears to be highly dependent on the limit of detection of the method being used. Measurement of eACTH using assays with a detection limit >5 pg/mL (considering an undetectable concentration as consistent with a functional AT) results in the incorrect classification of the cause of HAC in 15–26% of cases (Feldman, 1983b; Scott-Moncrieff et al., 2003; Zeugswetter et al., 2013). By contrast, using a chemiluminescent immunoassay with a limit of detection of 5 pg/mL, no overlap was observed between dogs with PDH (n = 91, range 6–1250 pg/mL) and dogs with functional AT (n = 18, all values <5 pg/mL; Rodríguez Piñeiro et al., 2009). However, in another study using an immunoradiometric assay with the same limit of detection, one (4.5%) of 22 dogs with PDH and one (16.7%) of six dogs with a functional AT were misclassified (Gould et al., 2001) with eACTH concentrations of <5 and 76 pg/mL, respectively. Possible explanations for this misclassification include delay in separation of plasma (for the first case) or the presence of concurrent PDH and functional AT (for the second case).

A high eACTH concentration in the absence of a pituitary tumour and undetectable eACTH concentration in the absence of an AT were also reported in a case of ectopic ACTH secretion (Galac et al., 2005) and food-dependent HAC (Galac et al., 2008), respectively.

Adrenal imaging

Imaging is often performed to complete the investigations for presenting signs, to add further support to a suspicion of HAC, or to evaluate the size, invasive nature and presence of metastases in dogs with ATs (Alenza and Melian, 2015). Imaging can also be used to differentiate between PDH and functional ATs in affected dogs (Behrend et al., 2013).

Adrenal glands are measured by width (mediolateral dimension), length (distance from cranial to caudal extremity) and thickness (dorsoventral dimension). In practice, thickness is the measurement most frequently used to evaluate adrenal gland size with ultrasonography, because it is a more accurate representation of gross measurements (Grooters et al., 1995), and its measurement was shown to have the lowest intra- and inter-observer variability as compared to length or width (Barberet et al., 2010). An adrenal gland thickness greater than 7.4 mm has nominally been considered consistent with adrenal enlargement (Barthez et al., 1995). However, normal adrenal size varies with breed, body size and age (Douglass et al., 1997; de Chalus et al., 2013; Bento et al., 2016). Caudal pole thickness of the left and right adrenal glands have upper thresholds of the reference intervals of 7.9 mm and 9.5 mm, respectively, in Labrador retrievers. By contrast, upper thresholds of 5.4 and 6.7 mm, respectively, have been established in Yorkshire terriers (de Chalus et al., 2013). In another study, the upper thresholds established in dogs ≤12 kg, and >12 kg were 0.62 and 0.75 cm, respectively (Bento et al., 2016). Dogs with PDH are expected to have bilateral adrenal enlargement and adrenal asymmetry is expected in dogs with functional AT. However, in practice, asymmetry of shape or size between glands does not necessarily indicate neoplasia. Indeed, in dogs with PDH, adrenal glands have been reported to be symmetrical and normal sized or enlarged but also mildly asymmetric in some cases (Benchekroun et al., 2010; Alenza and Melian, 2015). Additionally, one or multiple masses can be observed in dogs with macronodular adrenal hyperplasia with PDH. Complicating further the differentiation between functional AT and PDH with imaging, ATs are not always observed ultrasonographically. The right adrenal gland is notably more difficult to identify, particularly in large or overweight dogs, because of its more cranial and deeper location and smaller size compared to the left. Additionally, the presence of gas in the caecum may impair its identification. Compiling the data from several studies, in 71 dogs with a total of 79 ATs, 11 (14%) tumours were not identified ultrasonographically (Kantrowitz et al., 1986; Poffenbarger et al., 1988; Voorhout et al., 1990; Reusch and Feldman, 1991; Ford et al., 1993; Besso et al., 1997; Liste et al., 1997; Hoerauf and Reusch, 1999; Behrend and Kemppainen, 2001). However, some of these studies were performed more than 30 years ago. Since those early studies were performed, improvements in equipment and increased operator experience, may have significantly improved the likelihood of identifying ATs with ultrasonography.

Using ultrasonography, measurement of the smaller adrenal gland appears to best differentiate dogs with functional ATs from dogs with PDH. A maximal thickness of the smaller adrenal gland <5 mm was associated with a sensitivity of 100% and a specificity of 96% to identify functional ATs (Benchekroun et al., 2010). Contrast-enhanced ultrasonography is a non-invasive method for quantifying adrenal gland vascular patterns and may provide additional value to determine the origin of HAC. Peak perfusion intensity, adrenal blood flow and blood volume, as determined by contrast-enhanced ultrasonography, have been shown to be greater and time to peak has been shown to be longer in dogs with PDH as compared to healthy dogs (Bargellini et al., 2013; Pey et al., 2013). Additional studies in a population of dogs with HAC, including dogs with functional ATs, are required to evaluate the usefulness of this modality as a differentiating test.

Some structural features of adrenal masses can increase the suspicion for malignancy. Indeed, ATs larger than 2 cm in diameter are more likely to be carcinomas (Labelle et al., 2004). However, maximal adrenal dorsoventral thicknesses up to 3.7 cm were reported in dogs with adrenal hyperplasia (Benchekroun et al., 2010). Therefore, malignancy should not be assumed based on ultrasonographic measurements alone. Other ultrasonographic features supporting neoplasia include irregular adrenal enlargement and vascular invasion (Besso et al., 1997; Pagani et al., 2016).

Computed tomography (CT), particularly using reformatted images, may also help distinguish the cause of HAC. With reformatted images, an adrenal diameter ratio (maximal diameter of the larger gland/maximal diameter of the smaller gland) >2.08 had a sensitivity of 100% and a specificity of 98% for functional ATs in one study, including 46 dogs with PDH and 18 dogs with functional AT (Rodríguez Piñeiro et al., 2011). In practice, CT is rarely necessary for differentiation because of the availability of less expensive tests. Nevertheless, CT is often performed in dogs with ATs as part of staging or for surgical planning.

Pituitary imaging

Advanced imaging of the brain is performed in dogs with HAC if radiotherapy of a pituitary tumour or hypophysectomy is contemplated, to complete the investigations in dogs with discordant results in differentiating tests, or if neurological signs are present (Behrend, 2015).

Pituitary gland enlargement (pituitary height to brain area ratio $>0.31 \times 10^{-2}\,mm^{-1}$ assessed using contrast-enhanced CT) was only present in 43–70% dogs with PDH (Kooistra et al., 1997; Rodríguez Piñeiro et al., 2011; van Bokhorst et al., 2019). This poor sensitivity may be related to the poor soft tissue resolution of this imaging modality and the large proportion of microtumours in dogs with PDH. Other pituitary imaging modalities include dynamic CT and MRI, in which the flow of contrast medium into the pituitary gland can be visualised. Both techniques have shown promise in detecting extremely small pituitary tumours in humans (Stadnik et al., 1994; Friedman et al., 2007; Kinoshita et al., 2015). The pituitary gland appeared abnormal using dynamic CT in 45 of 55 (82%) dogs with known PDH (van der Vlugt-Meijer et al., 2003). In another study, an abnormal pituitary gland using dynamic CT (single slice dynamic scanning) was found in 30 of 49 (61%) dogs with confirmed PDH and without pituitary gland enlargement, as assessed by native-phase CT (van Bokhorst et al., 2019).

Conclusions

Diagnosis of HAC remains complicated, as there is no reference standard diagnostic test. The majority of studies evaluating the diagnostic performance of adrenal function tests have important shortcomings, notably because of the populations of dogs compared do not reflect those that would be evaluated in clinical practice. A few recent studies have attempted to include more representative cases and probably provide more realistic diagnostic performance indicators. Independent of the test used, maximizing diagnostic performance relies on testing dogs likely to have the disease (i.e., those animals that have consistent clinical and clinicopathological abnormalities). Future research should focus on evaluating the impact of various combinations of observations on the likelihood of HAC in individual cases. A diagnostic tool considering the signalment, clinical and clinicopathological features and adrenal function test results is currently being developed and may enhance the ability to more reliably diagnose HAC in the future (Graham et al., 2018).

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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