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The use of anti-Müllerian hormone as diagnostic for gonadectomy status in dogs

Axel P.N. Themmen [a,*], Bhanu Kalra [b], Jenny A. Visser [a], Ajay Kumar [b], Gopal Savjani [b], Jeffrey de Gier [c], Scott Jaques [d]

[a] Section of Endocrinology, Department of Internal Medicine, Erasmus MC, Rotterdam, The Netherlands
[b] ANSH Labs, Webster, Texas, USA
[c] Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands
[d] Texas A&M University Veterinary Medical Diagnostic Laboratory (Endocrine Section), College Station, Texas, USA

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ABSTRACT

In the veterinary practice, there is a need for a diagnostic tool to check the gonadal status in female dogs because it may be difficult to determine whether a female animal has been spayed or whether there are ovarian remnants. Although less prevalent, a similar situation pertains to male dogs. Anti-Müllerian hormone (AMH) is an important regulator of gonadal function and is a specific gonadal product that can be determined in circulation. The objective of this study was to develop and test a canine blood AMH assay as a diagnostic tool to determine the presence of functional gonadal tissue in dogs. A prospective study with a training-validation set paradigm was used. A canine AMH assay was developed and serum and plasma AMH concentrations were determined in blood samples from 46 intact female dogs, 48 spayed females, 50 intact males, and 48 castrated males collected at two separate institutes. Using a training-validation set paradigm, it was found that using cutoff values of 1.1 ng/mL (female) and 5.5 ng/mL (male) AMH, the assay reported excellent specificity and sensitivity of 100% and 90% in female dogs, and good specificity and sensitivity of 100% and 76%, in male dogs, respectively. The sensitivity in male dogs could be further enhanced by including a serum testosterone determination. This newly developed canine AMH assay is a valuable diagnostic tool to determine gonadal status in veterinary medicine.

1. Introduction

In veterinary practice, the presence or absence of functional gonadal tissue in dogs is a recurrent challenge awaiting a discerning diagnostic tool [1]. In particular, when the reproductive history is not known, it may be difficult to determine whether a female animal has been spayed. In female dogs, the presence of remaining functional ovarian tissue after spaying is relevant when a presumably spayed animal is presented with clinical signs of gonadal hormone activity such as vaginal discharge or behavior consistent with being in heat [2]. This is especially pertinent in cases when serum or plasma gonadal hormone values and vaginal cytology or vaginoscopy indicate anestrus. In addition, surgery performed at a young age or laparoscopic ovario(hyster)ectomy renders the visibility of surgical scars much more difficult. Notwithstanding the relative ease by which the presence of testicular tissue can be discerned by palpation in male dogs, cryptorchidism may result in a faulty diagnosis based on this technique [3]. Because gonadal function is regulated by the hypothalamic-pituitary-gonadal axis, the hormones that are involved in this axis have been proposed by various authors as possible markers to determine the state of

* Corresponding author. Tel : +31-10-7043340; fax: +31-10-7044862
E-mail address: a.themmen@erasmusmc.nl (A.P.N. Themmen).

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gonadectomy in dogs. De Gier et al., [4] have investigated whether GnRH stimulation of the hypothalamic-pituitary gonadal axis would allow the discrimination of intact or gonadectomized animals on the basis of plasma hormone determinations. These authors concluded that the LH:testosterone and the FSH:estradiol ratios had the best discriminatory power to differentiate intact from gonadectomized male and female dogs [4]. The gonadotropins LH and FSH, however, are more difficult to measure reliably than testosterone and estradiol. In male dogs, a single testosterone determination appeared to reliably verify castration status [4]. In female dogs, it was proposed that GnRH-induced estradiol is a reliable marker for functional ovary status [5], although this could not be confirmed [4]. The sex steroid hormone progesterone cannot discriminate between spayed and anestrous female dogs [5], thus making a second visit of the animal more likely based on estrus behavior. Such a scenario makes the identification of a better indicator of gonadal status in the female attractive.

A candidate hormone that could be used to determine gonadal status is AMH, a member of the transforming growth factor β family of growth and differentiation factors [6]. Produced exclusively in testicular Sertoli cells and ovarian granulosa cells, the physiological role of AMH in the two sexes is quite distinct [7,8]. In the male, AMH has an essential role in sex differentiation. Fetal Sertoli cells secrete AMH which signals the active removal of the Müllerian ducts, the anlagen of the oviducts, uterus and the upper part of the vagina in females, thereby preventing the formation of these structures in the male [9]. In postpubertal males, AMH has a role in the regulation of testosterone production by the Leydig cells. A negative correlation between testosterone and AMH has been found in human males [10], and it appears that testosterone suppresses Sertoli cell AMH production [11–13]. In females, AMH is not produced by the fetal ovary, ensuring that the Müllerian ducts stay intact in developing females. However, after growth initiation (recruitment) of primordial follicles, their granulosa cells start to produce AMH and continue to do so, until follicular selection by FSH has taken place. Anti-Müllerian hormone appears to have two roles in ovarian physiology. Firstly, AMH has a negative feedback effect on primordial follicles, that is, it suppresses recruitment thereby signaling the presence of a sufficient number of small growing follicles to the primordial follicle pool [8,14]. Secondly, AMH suppresses the sensitivity of the follicle to FSH in an autocrine manner, preventing selection [15]. While small follicles grow and differentiate AMH starts to decrease and when the differentiation state reaches the point when successful FSH selection is imminent, FSH sensitivity increases and the follicle is selected. Diagnostically, AMH is used to indicate the presence of testis tissue in patients with disorders of sex development and as a measure of the size of the ovarian reserve in women attending the fertility clinic [7,16].

The availability of AMH assays for dogs renders AMH as an attractive marker in the diagnosis of the gonadal status in dogs of both sexes, and AMH determination may offer advantages over the use of LH, FSH, testosterone, and progesterone. As previously mentioned, LH and FSH are difficult to determine reliably because the pulsatile secretion pattern necessitates GnRH stimulation and collection of multiple samples over time, whereas progesterone cannot distinguish between anestrous and spayed dogs. In addition, testosterone may also be produced by other tissues besides the testes, such as the adrenal glands [17]. In all mammalian species tested until now, AMH is exclusively produced by the granulosa cells of small growing follicles in the ovary and the Sertoli cells in the testis. In male dogs, immunohistochemical expression in testicular Sertoli cells has been reported [18]. It is expected that also in the dog ovarian granulosa cells express AMH, although the evidence in female dogs is lacking. Thus, AMH in neutered males would indicate the possible presence of functional normal or tumorous Sertoli cells. Similarly, presence of circulating AMH in a presumably spayed animal would indicate the presence of functional ovarian tissue or another source of AMH such as a granulosa cell tumor. In the present article, the use of serum AMH in the determination of functional gonadal status was investigated in intact and gonadectomized male and female dogs.

2. Materials and methods

2.1. Canine AMH assay

The canine AMH ELISA assay was developed using monoclonal antibodies (mAb) 37/4 and 37/7. The antibodies were developed by immunizing female AMH-deficient mice with recombinant human AMH (BA-047; Ansh Labs, Webster, TX, USA) and sorted on the basis of the affinity to different regions of AMH molecule as described previously [13]. The antibodies were epitope mapped using 80 overlapping biotinylated peptides across the precursor AMH [13]. Animal care and immunization were conducted in accordance with established guidelines and protocols approved by the Ansh Labs Animal Ethics Committee. Antibody 37/4 was coated on to a polystyrene microtiter plate (Greiner bio-one, Germany, cat # 705071), and antibody 37/7 was biotinylated using NHS-LC-Biotin (Thermo Scientific, USA, cat # PI21336) as described previously [13]. Pairing of the two antibodies was performed on recombinant human (Ansh Labs, BA-047), rat (Ansh Labs, BA-053) 140-kDa promature AMH, canine, bovine, and equine serum samples. No indications of breed-specificity were found (66 breeds tested).

2.2. Calibration

Calibrators were made in protein-based buffer using recombinant rat promature AMH preparations (Ansh Labs, BA-053). The assay uses a seven-point calibration (0.2–15 ng/mL, with blank subtracted). The log of AMH concentration is plotted on the X-axis, the log of matched optical density on the Y-axis, and the curve is fit using third degree polynomial regression (BioTek, Gen V, version 2.0, USA).

2.3. Assay procedure

The canine AMH assay is an enzymatically amplified two-site ELISA. In the first step, 50 μL of calibrators (15 ng to 0.23 ng/mL), controls, samples, and 50 μL of assay buffer

(protein-based TRIS buffer) were added to anti-AMH antibody coated wells and incubated with shaking at room temperature for 2 hours. Then, the wells were washed and 100 μL of the AMH antibody-biotin conjugate (mAb 37/7 in protein-based Tris buffer) was added to each well, and incubated with shaking for 1 hour at room temperature. Next, the wells were washed and 100 μL of streptavidin labeled horseradish peroxidase enzyme conjugate (Prozyme, Hayward, CA, USA cat # CJ30H) was added to each well and subsequently incubated with shaking for an additional 30 minutes at room temperature and washed. Hundred microliters of tetramethylbenzidine substrate solution (Neogen Corp., Lexington, KY, USA, K-Blue Aqueous tetramethylbenzidine Substrate, cat # 331199) was added to each well and then incubated with shaking for 8–10 minutes. The color formation was stopped by addition of 100-μL stopping solution (0.2-M H_2SO_4) to each well. The absorbance in the wells was read on a BioTek plate reader (BioTek instruments, Gen V, version 2.0, USA) at 450 nm as primary test filter and 630 nm as primary reference filter. Calibrators were used to plot a calibration curve of absorbance versus AMH concentration. The AMH concentrations in the samples were then interpolated from the calibration curve.

2.4. Assay analytical characteristics

To determine the analytical sensitivity, 16 replicates of calibrator A (0 pg/mL) and 16 replicates of calibrator B (0.2 pg/mL) were run in the canine AMH assay. The mean value plus two standard deviation of 16 replicate determinations of calibrator A (0 ng/mL) and 16 replicate determinations of calibrator B (0.2 ng/mL) were taken as the analytical sensitivity of the assay.

2.5. Linearity of dilution

Four young male canine samples with AMH concentrations in the range of 21–392 ng/mL were diluted in the calibrator A/Sample diluent. Multiple dilutions of these samples were then assayed against the calibration curve and the observed results were plotted against the expected value. Expected values were calculated by dividing the concentration from the undiluted sample by the dilution factor used. Percentage recovery was calculated by dividing the observed values by the expected values and multiplying by 100.

2.6. Imprecision

Imprecision of the assay was determined on two canine serum samples and the two kit canine serum controls with AMH concentration of 0.79, 2.40, 3.16, and 8.25 ng/mL, respectively. These samples and controls were run in quadruplets over 12 runs. Precision was expressed as percent coefficient of variation (%CV) for within run, between run and total assay ($n = 48$) variability.

2.7. Progesterone and testosterone assay

For the determination of the steroid hormones progesterone and testosterone, the Siemens Chemiluminesence (Immulite 2000) system was used. For progesterone, the interassay variation was 6.2%, and the intraassay variation was 4.8%. With respect to testosterone, the numbers were as follows; interassay variation 6.6% and intraassay variation 5.2%.

2.8. Immunohistochemistry

For immunohistochemical staining, sections were mounted on 3-aminopropyltriethoxysilane-coated slides (Sigma–Aldrich Chemie BV, Zwijndrecht, The Netherlands). After deparaffinization, sections were quenched for 20 minutes in 3% H_2O_2/methanol solution to block endogenous peroxidase activity, washed with water, and transferred to PBS. Sections were subjected to heat-induced antigen retrieval for 3×5 minutes at 700 W in 0.01-m citric acid buffer, pH 6.0 (Merck, Darmstadt, Germany), in a microwave oven, cooled down to room temperature, rinsed in PBS, subsequently incubated with a biotinylated AMH mouse monoclonal antibody (5/6A, Cat #: MCA2246; Serotec, Germany) [19], and diluted 1:100 at 4 C overnight followed by a wash step with PBS. Next, sections were incubated for 30 minutes at room temperature with streptavidin-biotin-peroxidase complex (ABC; diluted 1:200 in PBS; Dako, Glostrup, Denmark) and washed three times with PBS, and the peroxidase activity was developed with 0.07% 3,3′-diaminobenzidine tetrahydrochloride (Sigma–Aldrich). Finally, all sections were counterstained with hematoxylin. The immunohistochemistry control sections underwent the same protocol with the omission of the incubation with AMH biotinylated antibody 5/6A and reported no signal.

2.9. Collection of samples

Samples were collected at two separate institutes. At the out-patient clinic of the Faculty of Veterinary Medicine in Utrecht, The Netherlands, consecutive plasma samples of male and female dogs were collected in heparin-coated tubes. Samples were centrifuged at $2000 \times g$ for 5 minutes after which the plasma was collected and stored at 4 C for maximally 7 days until freezing and storing at −20 C. The College Station set of samples consists of serum samples submitted to the Texas A&M University Veterinary Medical Diagnostic Laboratory, College Station, TX, USA (AMH assays performed by Applied BioSciences College Station, TX, USA) for routine hormone analysis. Animals were selected on the basis of history of being intact or spayed/neutered from the submitting veterinarian/clinic. Exclusion criteria were unknown gonadal status or gonadal disease, whereas cycle stage of the female dogs, where determined, was not used. Two animals were excluded from the castrated male and intact female groups, because of underlying gonadal disease [20]; cryptorchid testis and granulosa cell tumor, respectively. This resulted in 46 intact female dogs (Utrecht: 17; College Station: 29), 48 spayed females (18, 30), 50 intact males (20, 30) and 48 castrated males (18, 30). Samples were stored at −20 C until assayed for AMH. Because the samples obtained from the Utrecht and College Station clinics were plasma and serum samples, respectively, we compared the AMH values of an independent set of 17 male and female dogs of which at the

same visit at the Utrecht or College Station clinics both serum and plasma samples were collected. Plasma AMH was found to be 0.66-fold of serum AMH ($r^2 = 0.89$; $P < 0.0001$). Plasma values were converted into their serum equivalents through division by 0.66, and the derived values were used for statistical analysis. Hereafter, we refer to all AMH values as serum AMH. This study was performed in compliance with the institutional guidelines for research on animals.

2.10. Statistics

The software package Graphpad Prism v5.01 (Graphpad Software Inc., La Jolla, CA, USA) was used to perform Student *t* test analysis. Receiver operator characteristic (ROC) analysis was performed using the software package MedCalc, v16.4.3 (MedCalc Software bvba, Ostend, Belgium).

3. Results

On screening of different preparations of the AMH molecule, the antibodies AMH 37/7 and AMH 37/4 reported strong affinity to recombinant 140-kDa human and rat promature AMH complex. The epitope mapping of the antibodies to different parts of AMH molecule revealed a heterogeneous epitope for 37/4 with strong bindings at amino acids (aa) 267 to 278, aa309 to 320, aa365 to 383 and 428 to 439, whereas antibody 37/7 reported a weak binding in the mature region at (aa484–495). The assay detected AMH in serum from human, equine, bovine, and all canine breeds. The analytical sensitivity of the assay as calculated by interpolating the mean plus two standard deviation of 16 replicates of calibrator A (0 ng/mL) and calibrator B (0.2 ng/mL) was 0.055 ng/mL. Values obtained in the assay below and above the lowest and highest calibrators (0.2 and 15 ng/mL, respectively) were reported as such, that is, at 0.2 and 15 ng/mL, respectively. Canine serum samples in the range of 21–392 ng/mL AMH diluted in the sample diluent reported linear results across the dynamic range of the assay (slope: 0.97; intercept: −0.11 ng/mL; $R^2 = 0.99$, $P < 0.001$). Reproducibility of the canine AMH assay was determined using two canine serum pools at 3.16 and 8.25 ng/mL and two kit controls (C1 and C2) at 21–392 ng/mL. The total imprecision calculated on serum pools and C1, C2 over 12 assays ($n = 48$) was 3.9 and 3.6% and 6.6 and 4.4%, respectively.

As stated in the introduction section, clear evidence of granulosa cell expression of AMH in female dogs is lacking. We therefore performed immunohistochemical analysis of AMH expression on sections derived from an ovary obtained after elective ovariectomy at the Department of Clinical Sciences of Companion Animals, Utrecht University. Because the antibodies that were selected for optimal performance in the canine AMH assay did not perform very well in the immunohistochemical analysis, possibly as a result of the different exposure of the AMH antigen caused by the fixation of the ovarian tissue, we decided to use a general AMH antibody which has been used in earlier studies on a variety of species, including humans [19,21]. Anti-Müllerian hormone expression is limited to granulosa cells of growing follicles (Fig. 1), whereas primordial follicles are devoid of antibody staining (not shown). Anti-Müllerian hormone expression in larger, antral follicles is more pronounced in granulosa cells closest to the oocyte.

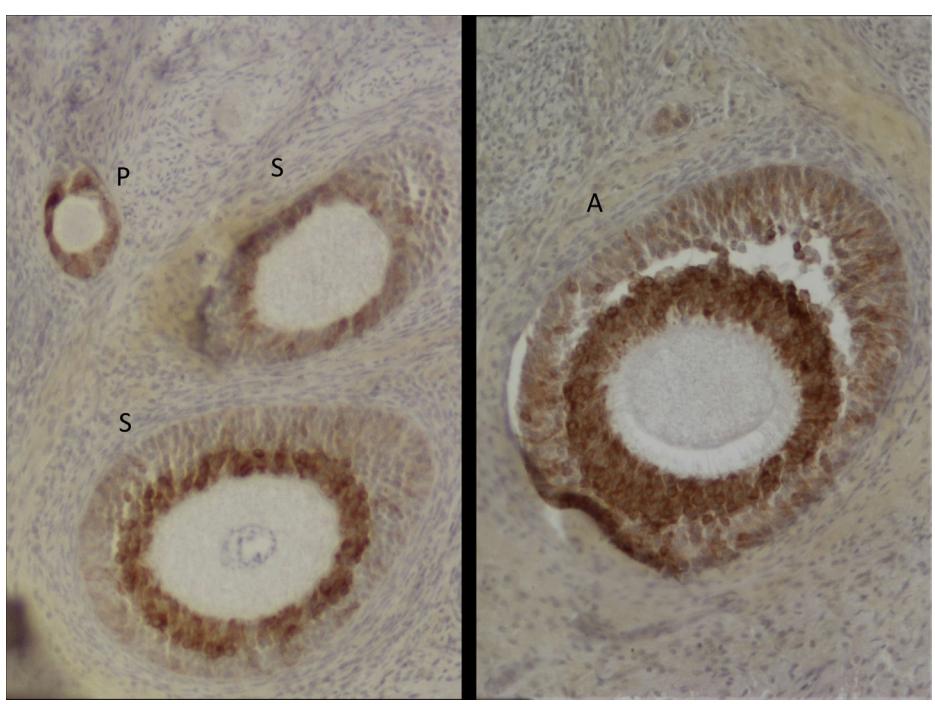

Fig. 1. AMH immune-reactivity in granulosa cells of primary (P), secondary (S), and a small antral (A) follicle. Notice absence of AMH staining in the theca layer and in the interstitial tissue. Also, notice increased AMH staining in granulosa cells closer to the oocyte. Magnification: 100×. AMH, anti-Müllerian hormone.

Graaffian follicles and (remnants of) corpora lutea are negative (not shown). Thus, as in other species, AMH expression in the dog ovary is present in the small, growing follicles and disappears in preovulatory follicles.

In Table 1, the mean values for AMH, testosterone, and progesterone are given. The latter two hormone values were determined in the College Station laboratory only in view of their possible use as additional information to determine functional gonad status of the animals (N = 30; see below in the Results section). The combined Utrecht and College Station serum AMH concentrations of intact and spayed female and intact and castrated male dogs were significantly different; female dogs, 3.9 ± 2.7 versus 0.2 ± 0.1 (ng/mL; mean ± SD; $P < 0.0001$); male dogs, 13.2 ± 4.9 versus 0.4 ± 0.7 ($P < 0.0001$). Generally, intact female dogs had lower AMH concentrations than male dogs ($P < 0.0001$; see also below). The AMH values of the two laboratories separately are depicted as scatter plots in Figure 2A, B.

In the data from the animals collected at Utrecht (training set), one unexpected value was found. At the low end, one intact female shows an AMH value below the lowest calibrator value of the assay (0.20 ng/mL), a value not expected when a functional ovary is present. The College Station set (validation) shows a high outlier in the intact females and seven intact males with unexpectedly low AMH (below 0.2 ng/mL).

We decided to take a two-step analysis of the value of the AMH assay in the clinical diagnosis of functional gonadal status in female and male animals. To this end, the Utrecht data set was first used as a training set to determine the AMH cutoff values in the determination of spayed/castrated status. Subsequently, the College Station set was used as an independent validation set. Thus, ROC analysis was performed to obtain optimal cutoff values and their associated sensitivity and specificity values in the training (Utrecht) data set (Table 2). In line with the difference in AMH values found in intact female and male animals, an approximately 5-fold difference in the suggested cutoff value for the diagnosis spayed or castrated are found; less than 1.1 and less than 5.5 ng/mL AMH for females and males, respectively (Table 2). The ROC analysis shows a high AUC of 0.96 and 1.0 for females and males, respectively. The sensitivity and specificity of the diagnosis "spayed" are 100% and 94%; for the diagnosis "castrated" 100% and 100%, respectively. The cutoff values are indicated in Figure 2 by the horizontal dashed lines.

In the next step in the analysis, the identified AMH cutoff values were applied to the College Station data set of AMH values. All spayed animals were correctly identified as being spayed on the basis of the cutoff value (AMH 1.1 ng/mL) as determined with the training set, whereas three of 30 intact females were incorrectly called spayed, resulting in excellent sensitivity and specificity of 100% and 90%, respectively. In the case of the male animals, all castrated males were called correctly (100% sensitivity), whereas 23 of 30 intact animals were correctly diagnosed as castrated (specificity 76%).

The incorrect calls prompted us to explore whether the progesterone and testosterone values could be used to increase the specificity values, especially in the case of the male intact dogs. In addition, we referred back to the animal history or contacted the owners or referring veterinarians. In the case of the seven incorrectly called intact males, it turned out that two animals had been incorrectly referred as intact, but rather had been castrated with corresponding concentrations of testosterone below the level of detection. A third animal was found to be diagnosed with Cushing's syndrome which may be accompanied by increased adrenal androgens and suppressed AMH [10]. Two animals had low, undetectable concentrations of testosterone (<0.20 ng/dL). One male remains unexplained with an AMH level below 0.2 ng/mL and testosterone of 303 ng/dL. One intact female was found to have been spayed on recheck. Exploring the progesterone data for the female dogs did not result in an improvement of the diagnostic specificity. All three intact females with a low AMH had progesterone values (0.36, 0.59, and 5.21 ng/dL) above the mean of the spayed group (0.24 ng/mL). Removing these animals from the analysis obviously would improve the specificity of the AMH diagnostic assay.

Table 1
Serum AMH, progesterone, and testosterone levels of the two sets of dog serum samples.

Sample origin	AMH [ng/mL]			
	Female intact	Female spayed	Male intact	Male castrated
Utrecht	4.3 ± 2.6	0.3 ± 0.2	15.0 ± 0.1	0.7 ± 1.1
College Station	3.6 ± 2.8	0.2	11.6 ± 6.4	0.2
Combined	3.9 ± 2.7	0.2 ± 0.1	13.2 ± 4.9	0.4 ± 0.7
	Progesterone [ng/dL]		Testosterone [ng/dL]	
	Female intact	Female spayed	Male intact	Male castrated
College Station	3.5 ± 5.9	0.24 ± 0.10	127 ± 114	2.0 ± 7.1

Abbreviation: AMH, anti-Müllerian hormone.

4. Discussion

The goal of the present study was to determine the effectiveness of an AMH measurement as a diagnostic for the presence of functional gonadal tissue in dogs. To this end, we developed a highly specific AMH assay and reported that, as in other species, ovarian AMH expression is limited to the granulosa cells of small and antral growing follicles. Using a training-validation paradigm, we found that a single serum AMH determination predicts with 100% sensitivity and 90%–76% specificity the absence of functional ovarian or testicular tissue, respectively. Inclusion of serum testosterone in the diagnosis of the male animals would increase the specificity.

The canine AMH assay that was developed in the present study shows excellent test properties. This may be the result of the technique used to raise highly specific monoclonal mouse AMH antibodies, that is, using a purified human recombinant AMH preparation in mouse AMH null mice. The combination of a pure preparation and the naivety of the mouse immune system to AMH resulted in a high number of successful hybridomas producing antibodies with excellent affinities as previously shown which

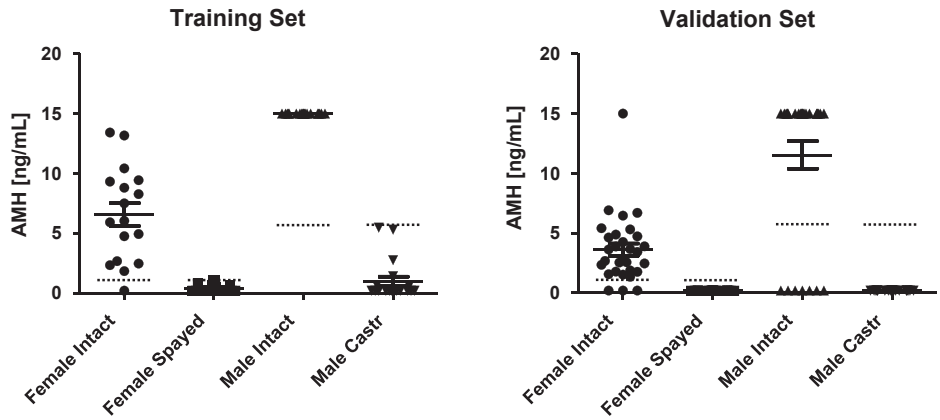

Fig. 2. Scatterplots of individual serum AMH values in dogs. Training set: AMH values of the serum samples collected in the Utrecht University clinic. These values were used to determine optimal cutoff values of 1.1 and 5.5 ng/dL AMH in females and males, respectively. The cutoff is indicated by the dashed line. Validation set: AMH values of the serum samples collected in College Station. These values were used to validate the cutoff values of the canine AMH assay that are indicated by the dashed lines. AMH, anti-Müllerian hormone.

allowed careful selection of the best high-affinity and species-specific antibodies [22]. Because antispecies antibodies can cause interference in immunoassays by generating false positive results and have been reported to be found in anywhere from 1%–80% of a population [23], heterophilic blockers were used in the assay to minimize the false positives. Importantly, a set of internal standards and controls was prepared and stored for future reference to minimize the lot-lot variability over time. A well-characterized purified recombinant canine AMH preparation (international reference preparations) will be required to standardize the test and minimize the inter method bias.

Although the antibodies did not prove to be suitable for use in immunohistochemical analysis of AMH expression in dog ovarian tissue, we were able to show that in the ovary, AMH expression is limited to granulosa cells of the small growing follicles. The pattern of follicular expression appears to be similar to that found in other species, including humans [19,21,24,25]. Anti-Müllerian hormone expression is lost when follicles increase in size and are further developed and differentiated toward the state where selection by FSH takes place. The overall pattern is that AMH expression in the granulosa cells most closely associated with the oocyte is lost last during follicular growth and differentiation [26,27]. As illustrated in the present article, this is also the case in the dog ovary, in which the separation between granulosa cells expressing or nonexpressing is even more pronounced than in other species. Furthermore, the follicular expression pattern shows that small growing follicles express AMH which are also present in anestrous female dogs [28], thus indicating the usefulness of serum AMH in each stage of the estrous cycle and is not limited to estrous as also indicated by the findings of Place et al. [1].

Table 2
Receiver operator characteristic analysis of prediction of the diagnosis "spayed" and "castrated" by AMH determination (Training set).

Sample origin	Cutoff AMH (ng/mL)	Sensitivity	Specificity	AUC
Female	<1.1	1.0	0.94	0.96
Male	<5.5	1.0	1.0	1.0

Abbreviation: AMH, anti-Müllerian hormone.

The serum samples were collected from the normal practice as it exists in the university diagnostic laboratories. No systematic differences in values were found between different breeds and therefore dogs of all breeds were included in the study. Exclusion criteria were known gonadal disease because it is well known that Sertoli and granulosa cell tumors produce large amounts of AMH, which could bias the outcomes of the study [18,20].

In the analysis of the serum or plasma AMH determination as a diagnostic tool, we decided to take the training-validation cohort paradigm which has several advantages. Two independent cohorts were studied collected at different university diagnostic laboratories located in different continents, that is, Europe and the USA. One cohort was used for the development of the serum AMH cutoff values with the optimal sensitivity and specificity, which could subsequently be tested in the second independent collection of animals for their diagnostic performance. An exploratory ROC analysis of the validation cohort suggests that the AMH cutoff values could be lowered to 0.20 ng/mL (the detection threshold of the assay) for both sexes, with accompanying sensitivity and specificity of 100% and 77% for male dogs, and 100% and 90% for female dogs. The determination of the definitive cutoff values will only be possible on a future prospective sample collection. An additional advantage is that this paradigm allowed us to decide to determine the sex steroid hormones progesterone and testosterone as well because these latter hormones might be of additional value for the diagnosis of the presence of functional gonadal tissue. The sensitivity and specificity show that serum AMH determination can be proposed as the best available method to determine gonadal status in dogs. This is certainly the case in the female dogs, where no good alternatives exist and progesterone is not very useful when a low concentration is

found because values of this hormone are very low, both in spayed and anestrous phase animals.

The assay shows its usefulness by identifying those dogs that have been attributed wrongly with the diagnosis spayed/intact or castrated/intact, which resulted in the correction of the gonadal status label for two intact males and one intact female. Indeed, the presence or absence of gonads appears not always to be noted carefully in the history description resulting in an expected bias toward animals wrongly identified as intact. Thus, three of the four corrections were from intact to gonadectomized.

The sensitivity of the AMH test indicates that all neutered or castrated animals will be correctly identified. Thus, in cases when clients come to the veterinary clinic with unexplained sexual behavior in spayed or castrated animals, an AMH value above the cutoff indicates that the cause of aberrant behavior is likely to be the presence of functional gonadal tissue, eliminating unnecessary procedures for the investigation of remnant or occult gonadal tissue. Therefore, the determination of AMH is a minimally invasive choice in the differentiation of gonadal status.

Another application of the AMH test may be the screening of animals with unknown history such as at arrival at an animal shelter. Many such shelters have the policy that dogs are spayed or castrated before they are offered for adoption. If the present AMH assay would be used without any additional diagnostic tools, approximately 1:10 females and 1:4 males will be wrongly attributed to a gonadectomized status. However, in the male dog group, this problem may be not as large as it appears. First, testes are easily palpated and because this diagnostic tool would be applied before any blood tests are considered, palpation would increase the specificity of the AMH test. Nonpalpated, cryptorchid testes most probably will still produce AMH detected with the assay [29,30]. Second, the sensitivity could also be increased by the inclusion of a testosterone determination. Two of the five intact animals with AMH values below detection had testosterone values below 0.20 ng/dL suggesting impaired testis function although rechecking their history or checking with the referrals did not reveal any information to support this suggestion. The inclusion of a single testosterone determination may improve the performance of the AMH test when the determination of castration status is the goal. Testosterone without AMH as a single diagnostic test is not a good alternative to the AMH assay described in the present article. An ROC analyses of the testosterone data resulted in a cutoff of less than 35.2 ng/dL with a sensitivity of 100%, but a specificity of only 73% (likelihood ratio: 3.8), which is certainly not better that the AMH values by themselves. Most probably, a combination of the two hormones is the most optimal.

In the female dogs, the number of incorrectly identified intact animals is more difficult to diminish. As stated in the introduction during anestrus progesterone values are low and will not help to discern animals wrongly identified as being spayed. A single serum LH determination has been proposed, although such a measurement by itself has moderate specificity [31]. GnRH stimulation tests in intact anestrous and ovariectomized female dogs show that estradiol concentrations only increased in anestrous but not in ovariectomized dogs [5]. This was extended in a second study in female dogs 6 months after gonadectomy, which reported that the plasma estradiol concentration 120 minutes after GnRH stimulation could be used to discriminate between female dogs with or without ovarian tissue [4]. However, a GnRH stimulation test most likely will not easily be implemented in environments such as animal shelters in view of its complication and cost.

4.1. Conclusions

In conclusion, we have developed a canine serum AMH assay, which can be used with maximal sensitivity and optimal specificity to determine the gonadectomy status of male and female dogs. In male dogs, an additional single testosterone determination may increase the diagnostic properties of the test, whereas in female dogs; the added value of a GnRH stimulation test should be further investigated.

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B.K., A.K., G.S. are employees of ANSHlabs, Webster TX, USA. A.T. consults for ANSH Labs, Webster, TX, USA.

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