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

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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

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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