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Relative Accuracy of Diagnostic Methods in Verification of Gonadal Status of Dogs Considering Anti-Mullarian Hormone as a Biomarker

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Abstract

The study was carried out to demonstrate relative efficacy of various methods to ascertain gonadal status in dogs. Based on different diagnostic methods viz., history and clinical signs, physical examination and ultrasonography, 37 (21 male and 16 female) dogs were categorised into different groups. Group I- gonadectomised dogs (n=10; Male-5 Female-5), Group II - intact dogs before and after gonadectomy (n=16; Male-8 Female-8), Group III-dogs with ovarian remnant syndrome (ORS) (n=3) and Group IV- dogs with either unilateral/ bilateral cryptorchidism (n=8). All the dogs were subjected to serum Anti- mullarian hormone (AMH) estimation using canine specific AMH kits. The mean AMH level in intact male dogs, castrated and cryptorchid dogs was 16.20 ± 1.44, 0.57 ± 0.06 and 24.90 ± 0.79 ng/mL, respectively. In female dogs, the mean AMH value was 5.14 ± 0.54, 3.20 ± 1.54 and 0.26 ± 0.02 ng/mL in intact, ORS and spayed dogs, respectively. By considering AMH as the standard to ascertain the gonadal status, the relative accuracy of history and clinical signs was 85.71 and 100%, physical examination was 61.90% and 43.75%, whereas with trans- abdominal ultrasonography 71.42% and 18.75% in male and female dogs, respectively. A single serum AMH concentration measurement was highly effective in accurately distinguishing between gonadectomized and intact adult dogs. In addition, this test also accurately identified several cases of ovarian remnant syndrome and cryptorchidism which were failed to identify using other methods.

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INTRODUCTION

In veterinary practice, determining whether functional gonadal tissue is present in dogs is a frequent challenge, particularly when reproductive history is unclear. This difficulty is heightened in female dogs spayed at a young age or those that have undergone laparoscopic ovariectomy because these procedures often leave minimal or no visible surgical scars. [1]. The presence of remnant functional ovarian tissue becomes relevant when spayed animals present with vaginal discharge or proestrus/estrus behavior due to gonadal hormone activity [2, 3].

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The presence of scrotal testes may be easily determined by palpation in male dogs, but cryptorchidism may result in a faulty diagnosis. Gonadal function is regulated by the hypothalamic-pituitary-gonadal axis, and the hormones involved in this axis have been proposed by various authors as possible markers to determine gonadal status in dogs. GnRH stimulation allows the discrimination of intact or gonadectomized animals based on plasma hormone determinations. Some authors have opined that the LH: Testosterone and the FSH: Estradiol ratios have the best discriminatory power to differentiate intact from gonadectomized male and female dogs [4]. However, gonadotropins (LH and FSH) are more difficult to measure reliably than testosterone and estradiol because of their pulsatile secretion patterns and require repeated sampling. In male dogs, a single testosterone determination appeared to reliably verify the castration status, but testosterone can also be produced by the adrenal glands [4]. Although it was proposed that GnRH-induced estradiol is a reliable marker for functional ovary status in female dogs, it could not be confirmed [4]. The progesterone level is always at its basal level (< 1 ng/mL) during anestrus and up to proestrus, and cannot discriminate between spayed and anestrus female dogs, thus making a second visit to the animal more likely based on estrus behavior [5, 33]. In such situations, an appropriate hormone that can be used to determine gonadal status is Anti Mullerian Hormone (AMH), a dimeric glycoprotein and a member of the transforming growth factor β family of growth and differentiation factors produced exclusively in testicular Sertoli cells and ovarian granulosa cells, and the physiological role of AMH in the two sexes is quite distinct [6].

AMH is produced by granulosa cells of developing follicles (pre-antral and small antral) in the ovary, starting from the onset of folliculogenesis and continuing until reproductive senescence. As a result, it serves as a valuable endocrine marker for assessing the ovarian reserve throughout a female's reproductive life. Additionally, AMH is used to predict ovarian response to infertility treatments and potential pregnancy outcomes in women [7, 8, 34]. In production animals, AMH has been utilized as an endocrine marker to predict the pool of ovarian gonadotropin-responsive follicles in cows, goats, and ewes [9-11]. AMH is also regarded as a valuable clinical tool for selecting suitable donors for superovulation programs and for maximizing the number of transferable embryos in dairy cows. Additionally, it is used to predict the number of follicles available for oocyte aspiration programs in mares [12, 13].

AMH plays a critical role in sex differentiation in males. During fetal development, Sertoli cells secrete AMH, which signals active regression of the Müllerian duct system, preventing the development of female reproductive structures [14]. In post-pubertal males, AMH plays a role in regulating testosterone production by the Leydig cells., in females, the fetal ovary does not produce AMH, allowing the Müllerian ducts to remain intact during development. After the initiation of growth (recruitment) of primordial follicles, granulosa cells within these follicles begin to produce AMH, continuing until follicular selection by FSH occurs. AMH exerts a negative feedback effect on primordial follicles, suppressing their recruitment and signaling the presence of an adequate pool of small growing follicles to maintain the follicular reserve [7, 15]. AMH also reduces follicle sensitivity to FSH via an autocrine mechanism, thereby preventing premature selection. As small follicles grow and differentiate, the AMH levels begin to decrease. When the follicles reach a differentiation stage, where successful FSH selection is near, their sensitivity to FSH increases, allowing the follicle to be selected for further development [16].

AMH is exclusively produced by granulosa cells in small developing follicles in the ovary and Sertoli cells in the testis across all mammalian species studied. In male dogs, immunohistochemical studies have confirmed AMH expression in testicular Sertoli cells [17]. Consequently, the detection of AMH in neutered males may indicate the presence of either functional normal Sertoli cells or tumorous Sertoli cells [18]. In a seemingly spayed female dog, the presence of circulating AMH suggests the existence of functional ovarian tissue or granulosa cell tumor of the ovary [19]. To demonstrate the relevance of serum AMH estimation as well as the cut-off value of this hormone in determining functional gonadal status in intact and gonadectomized male and female dogs and in cases of ovarian remnant syndrome and cryptorchid dogs, the current study was carried out.

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MATERIALS AND METHODS

The dogs that presented to the outpatient ward of the college hospital between June 2021 and December 2021 for elective castration, routine ovariohysterectomy, dogs with cryptorchidism and ovarian remnant syndrome, and male and female dogs sterilized at least six months back in this clinic were considered for the study. The dogs were aged between 1.5 and 10 years, and the owner's consent was obtained before the collection of samples. This study was approved by the Institutional Animal Ethics Committee (VCH/IAEC/2021/40).

The owners were asked about the history of pets' sterilization; male dogs exhibiting any masculine behavior tended to become muscular and changes in the hair coat. Potential exposure of pets to exogenous hormones (hormone replacement therapies and anabolic steroids) was sought, and such dogs were excluded from the study. A thorough clinical and andrological examination was conducted, which included careful inspection of the penis and palpation of the inguinal region. The evaluation assessed the presence or absence of testes within the scrotum along with a digital rectal examination of the prostate in male dogs. Additionally, the pre-scrotal area was inspected for any visible surgical scars if present.

Female dogs with a history of vulvar swelling, bloody discharge, or attracting males in the intact state or dogs with ovarian remnant syndrome (ORS), which were already spayed, were recorded, including the age at which the dogs were sterilized. Detailed clinico-gynecological examination was performed to check for constriction or atrophy of the vulva and vagina, and abdominal palpation of the uterus was performed. The ventral abdomen and flank regions were carefully inspected for evidence of a surgical scar and atrophy of the mammary glands (Figure 1).

In dogs with a clear history of ovariohysterectomy, in which behavioral signs of estrus were present or suspected during the examination, vaginal cytology was conducted to assess the presence of cornified vaginal epithelial cells (Figure 2). Serum progesterone estimation was performed in previously spayed dogs after 21 days of showing signs of proestrus/estrus using an automated immunoassay system (M/s. Biomerieux Mini Vidas kit) based on enzyme-linked fluorescent assay (ELFA) principles.

All study dogs were subjected to scanning of the abdomen and inguinal area using a real-time B-mode ultrasound scanner with a linear array transducer equipped with a frequency of 3.0 to 8.0 (Prosound Alpha, Japan) for visualization of the retained testis (Figure 3a), descended testis (Figure 3c), and prostate size in male dogs (Figure 3b). In female dogs, transabdominal ultrasonography was performed to ascertain uterine enlargement (Figure 3d) or other pathologies.

Based on the history, physical examination, vaginal exfoliative cytology, progesterone level, and transabdominal ultrasonography, 37 (21 male and 16 female) dogs were categorized into four groups, and AMH was estimated in all dogs. **Group I** (n=10; male-5 female-5): gonadectomized dogs served as positive control, **Group II** (n=16; male-8 female-8): both male and female intact dogs before and after gonadectomy; **Group III** (n=3), dogs with ovarian remnant syndrome (ORS), and **Group IV** (n=8): dogs with either unilateral and/or bilateral cryptorchidism.

Blood collection for AMH estimation was performed at least six months after castration and ovariohysterectomy in male and female dogs of Group I, respectively, on days 0 and 10 of surgery in Group II dogs, which were subjected to routine castration and ovariohysterectomy. Blood was collected at the time of proestrus bleeding in dogs with ORS (Group III), on the day of tentative diagnosis of the conditions, and 10 days after orchiectomy in dogs with either unilateral or bilateral cryptorchidism (Group IV). The canine AMH enzyme-linked immunosorbent assay (ELISA) kit (ANSH Lab, Webster, Texas, USA) was used as a diagnostic tool for quantitative measurement of AMH in canine serum. This assay aids in determining the functional gonadal status of dogs, whether they are spayed, neutered, or intact.

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The AMH concentrations before and after gonadectomy in male and female dogs in group II were statistically analyzed by paired t-test using GraphPad Prism version 5.0. One-way way ANOVA was used to compare the significance between the mean AMH values of all groups. level of significance was set at 95%.

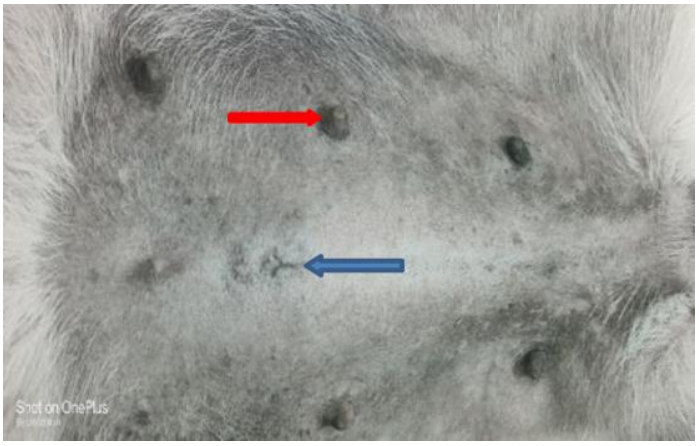


Figure 1. Surgical scar at mid-ventral abdomen (blue arrow) and Mammary gland atrophy (red arrow).

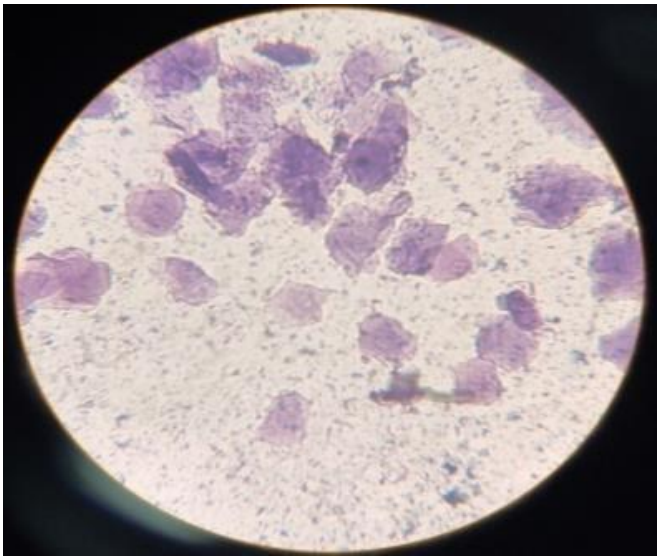
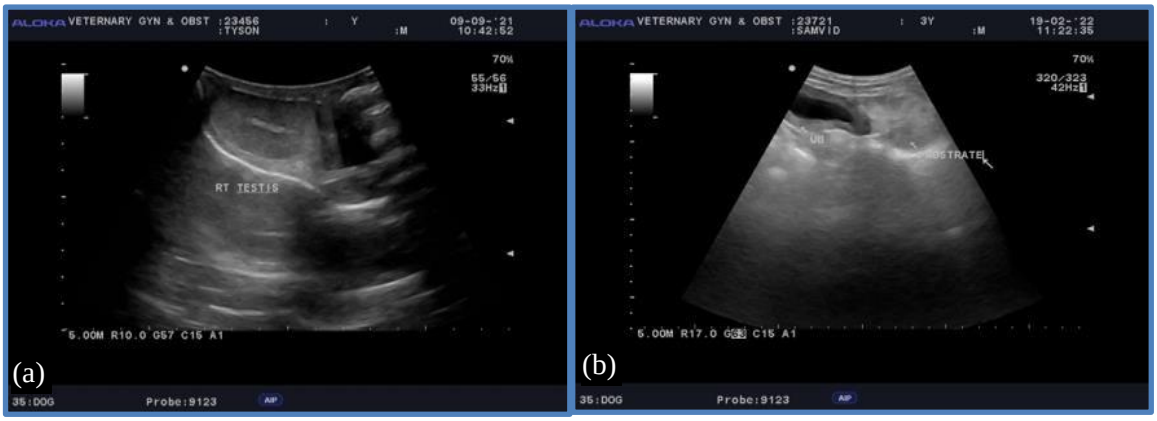


Figure 2. Cornified cells in exfoliative vaginal cytology (ORS).



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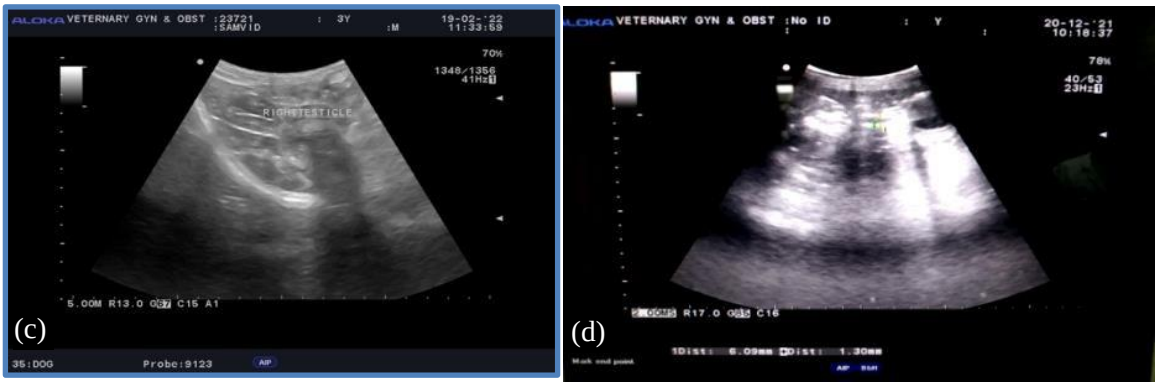

Figure 3. Ultrasonographic image (a) normal scrotal testis (b) Prostate gland (c) abdominal testis (d) uterus in diestrus stage of dog.

RESULTS AND DISCUSSION

No one wants an animal to undergo unnecessary anesthesia or surgery because of a lack of proper neutering history. When preparing to neuter a pet, it is crucial not only to accurately identify the sex of the dog or cat, but also to ascertain whether the animal has been previously spayed or castrated. A clinician with available techniques can verify the spay–neuter status of the patient before surgery, including a proper history from the owner, clinical signs, physical/clinical examination, ultrasonography, and hormonal evaluation. Hence, this study was carried out in 21 male and 16 female dogs to compare the relative accuracy of the above methods with AMH concentrations to ascertain the gonadal status of dogs.

History and Clinical Signs

All intact and gonadectomized dogs were identified on the basis of their history and clinical signs. Furthermore, another three dogs that exhibited proestrus signs even after 6 months post spaying were tentatively diagnosed with ovarian tissue (ORS). In contrast, five out of eight male dogs presented with a complaint/history of absence of scrotal testis but exhibited masculine behavior such as urine marking, mounting on female dogs, etc., and were diagnosed with cryptorchidism. However, three owners out of eight had failed to observe these signs or such male dogs did not get an opportunity to get exposed to female dogs especially during the proestrus/estrus and making difficult for a veterinarian to ascertain gonadal status (Table 1). Cryptorchid dogs exhibiting urine marking or spraying, fighting, female attraction, or mounting have also been reported by [20]. Cryptorchid/retained testes do not produce spermatozoa, but secrete testosterone. The absence of scrotal testicles causes infertility in dogs, but does not prevent the development of androgen-dependent behavior [21]. Such history/symptoms provided by the owners may suggest the presence of undescended testicles. Puppies are castrated at an early age and subsequently adopted by a person who may not observe any specific male behavior, thus making the clinician suspect cryptorchidism. Based on history and clinical signs, out of 21 male dogs in this study, 13 were identified to have intact testes and 5 dogs were castrated, with an overall accuracy of 85.71 per cent (Table 2).

Some bitches and queens presented to veterinarians and animal shelters with unknown histories underwent anesthesia and surgery only to reveal whether ovariohysterectomy (OHE) was performed. This situation can lead to trauma to the animal, increased expenses for the owner, and frustration for the attending veterinarian. Although surgical exploration is often the most effective approach for diagnostic purposes, it ensures that no female patient is left without ovariohysterectomy. Nonetheless, avoiding surgery is important for potentially “high risk” patients for anesthesia, such as morbidly obese and brachycephalic animals necessitating the accuracy of spay status determination in dogs. Among the 16 females, 11 were identified as intact (with ovary/ovarian tissue) and 5 as ovario-hysterectomized based on history and clinical signs, giving an accuracy of 100 per cent. Although history helps the clinician to determine the animal’s true spay status, the possibility of ovarian remnant syndrome in

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spayed dogs should not be discounted, as it may be encountered anytime between one and ten years following neutering. Hence, proper questionnaires to the owners to obtain data on display of proestrus/estrus behavior, marked enlargement of the vulva, and bloody discharge become imperative to recognize ORS. In such cases or when there are suspicions at the time of examination, vaginal cytology can be performed to confirm the presence of cornified vaginal epithelial cells. This should be performed after ruling out any potential exposure to exogenous hormones, such as hormone replacement therapy, which could explain the observed clinical signs [22].

Physical Examination

The physical characteristics of the external genitalia in dogs can vary significantly depending on the age at which the individual is neutered. Dogs neutered during puppyhood tend to have an infantile appearance of the penis and prepuce compared with those neutered at a more traditional age or as mature adults. Consequently, the presence of an infantile penis and prepuce in an adult dog without testicles serves as an objective indicator of a previous castration. Additionally, the presence of a surgical scar in the pre-scrotal area can help to confirm castration; however, this finding should be interpreted with caution, as such scars may also arise from unrelated skin trauma. [22]. Similarly, in the present study, three of the five male dogs were identified as castrated by clinical/physical examination, with evidence of a U-shaped left ear notch and markedly reduced prostate size on digital rectal examination, which was also reported by Atalan *et al.* (1999) [32]. Physical examination of the entire inguinal region (i.e., from the level of the inguinal rings to the scrotum) successfully confirmed two of the eight cases of cryptorchidism/ectopic testis. Retained testicles are generally grossly smaller than the scrotal testicles. Testicles located in the inguinal region are often difficult to palpate owing to inguinal fat deposits. Sertoli cell tumors (SCT) in dogs are frequently quite large compared to those in other abdominally retained testicles, and signs of feminization may also be apparent on physical examination.

In general, intact male dogs are typically larger and more muscular than neutered males. Additionally, changes in the hair coat often occur after neutering. However, these changes are highly subjective and can be influenced by various factors beyond neuter status. As a result, they may go unnoticed, especially if the owners are not deeply familiar with a specific breed. [23]. On physical examination, out of 21 male dogs, 10 were identified as intact and 3 as castrated, with an accuracy of 61.90 per cent. Although the dogs are very diverse physically, the vulva of some intact bitches appears small during anestrus, making it difficult to differentiate them from spayed dogs by physical/clinical examination, and some well-developed bitches experience dramatic vulvar atrophy after ovariohysterectomy. Clinicians must interpret these findings with caution, considering them in the context of all other relevant observations. Physical examination for evidence of a surgical linear scar aids in verifying the gonadectomy status but is not a reliable indicator because a prominent linea alba may sometimes be mistaken for a scar, and scars in spayed dogs are not readily palpable [23]. Moreover, the presence of a scar does not reveal spay status because ventral abdominal scars may be the result of cesarean section and other abdominal surgeries in some cases. Additionally, previously spayed animals may show no signs of an abdominal incision, particularly if the spay was performed at a very young age or using a flank approach or laparoscopic technique [1] was used. Therefore, clinicians must cautiously interpret this finding by considering all other findings, including history and physical examination, including mammary gland, vagina/vulva, etc., in order to make a proper assessment (Griffin, [22].

In cats and dogs spayed after puberty, the mammary glands become atrophied with very small teats and an atrophied vulva in contrast to intact females; therefore, careful examination of such changes is relevant [21]. Physical examination in the present study showed that 6 out of 16 were intact and one was ovario, with an accuracy of 43.75 per cent. However, female dogs exhibiting signs of proestrus with vulvar swelling need to be subjected to exfoliative vaginal cytology to confirm the presence of ovarian tissue. In this study, two of the three ORS cases (Group III) showed the presence of cornified anucleate or superficial vaginal epithelial cells, confirming ovarian activity. Circulating levels of estradiol induce changes in vaginal epithelial cells, which can be monitored using vaginal cytology.

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[24,25]. In dogs with a functional ovarian remnant, estrogen secretion causes progressive cornification of the vaginal epithelial cells and is considered the cheapest, easiest, and acceptable tool for the diagnosis of ORS [26].

Ultrasonography

Ultrasonography refers to the use of ultrasound technology to image and assess various aspects of animal health, reproduction, and anatomy. It is widely used in veterinary science and animal breeding for noninvasive diagnostic and monitoring purposes. On ultrasonography, the testicular parenchyma has a hypoechoic background structure overlaid by a homogenous medium echogenic stippled appearance with the mediastinum testis seen as a thin echogenic band aiding as a referral point in localizing the undescended testicle [27]. In the present study, all eight intact male dogs with scrotal testes and three cryptorchid dogs out of eight with the complaint of absence of scrotal testes were confirmed. In dogs and cats, congenital undescended testes can be located anywhere between the caudal pole of the kidneys and inguinal area [28]. Ultrasound is a sensitive diagnostic technique with a high positive predictive value to locate retained testes in companion animal by the hands of experienced operators. The sensitivity of ultrasound was 96.60 per cent for abdominal and 100 per cent for inguinal testes in a previous study [29]. In the present study, 11 male dogs were found to be intact and 4 were castrated among 21 male dogs; 3 out of 16 female dogs were identified as intact when subjected to transabdominal ultrasonography, with 71.42 per cent relative accuracy in ascertaining the gonadal status in male dogs and 18.75 per cent in female dogs.

Incomplete surgical removal of one or both ovaries occurs because of inadequate exposition of the ovaries, improper positioning of the hemostatic clamps or ligatures, and Arteriovenous Ovarian Complex (AVOCs) [30]. In these cases, collateral neovascularization of the remnant ovary may occur, even if hemostasis of the AVOCs is achieved [31]. A small abdominal incision can complicate the visualization and accessibility needed to effectively ligate the ovaries. Additionally, incorrect ligation of the ovarian tissues or the anatomical location of the right ovary are commonly accepted reasons for ovarian remnant syndrome (ORS). [26,20,37,38]. Occasionally, a fragment of the ovarian tissue may inadvertently fall into the abdominal cavity during ovariectomy. This tissue can revascularize with the omentum or serosa of the abdominal viscera and function similarly to a normal ovary [38].

In the present study, only in two out of eight intact dogs (Group II), the cervix and uterine horns were visualized as a continuous hypoechoic oval structure between the anechoic urinary bladder and the hyperechoic crescent-shaped colon as evidence of ovarian activity with the help of ultrasonography, followed by [39] for easy assessment of the uterus as an indicator of intactness. During proestrus and estrus, thickening of the uterine walls with luminal fluid due to estrogen-induced edema is evident and is due to the action of progesterone during diestrus, especially on transverse images. However, during anestrus, the ovaries and adjacent tissues will have similar echogenicity, making it very difficult to locate, while the uterine body will be flat and homogeneous, with no changes in the endometrium and absence of luminal content [39, 40]. Ultrasonographic evaluation of female genital system can be done using high frequency transducers of 5.0 to 10.0 MHz, however, lower frequency transducers are useful in assessing ovarian and uterine dimensions and also it could be difficult to image the ovaries/ovarian tissue especially during anestus [41, 42, 43]. Of the three ORS cases, one dog with stump pyometra was diagnosed using ultrasonography. However, the accuracy of this method is affected by several factors, including the size of the ovarian remnant, stage of the estrous cycle at the time of scanning, and expertise of the ultrasonographer [44].

Anti-mullarianHormone

The anti-Müllerian Hormone (AMH) is a protein hormone that plays a key role in reproductive biology. In animal biotechnology, AMH is particularly important for managing and optimizing reproductive performance, particularly in livestock breeding programs and genetic selection.

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The mean AMH level in the control male dogs and in male dogs 10 days of gonadectomy were almost similar (0.57 ± 0.06 vs 0.58 ± 0.05 ng/mL) whereas it was 16.20 ± 1.44 ng/mL in the intact male dogs in the present study. Similar AMH values of 13.2 ± 4.9 and 0.40 ± 0.70ng/mL before and 10th day post castration, respectively using canine AMH ELISA assay have been reported [34]. Lesser values of 1.27 ng/mL, 5.1 ng/mL [18] 5.63 ng/mL [35.2 ng/mL using either the human AMH kit or canine AMH kits by chemiluminescence assay have been reported in intact male dogs.[45] have reported a decreased median AMH value of 0.08 ng/mL in dogs after castration. [46] reported a mean AMH value of 6.49 ± 3.24 µg/L in intact male dogs using human AMH kits by ECLIA (Figure 4).

The AMH levels in the control female dogs and gonadectomized female dogs were similar (0.26 ± 0.18 vs 0.26 ± 0.02 ng/mL). The mean level of AMH in female dogs before and 10 days after gonadectomy was 5.14 ± 0.54 and 0.26 ± 0.02 ng/mL, respectively. These values are in agreement with those reported by Themman *et al.*

(2016) using canine specific AMH ELISA kit who have reported the mean AMH of 3.9 ± 2.7 ng/mL and 0.2 ± 0.1 ng/mL, respectively in intact and 10 days post spaying in female dogs.[49] also reported a similar mean AMH level of 4.26 ± 0.82 ng/mL in intact female dogs using canine specific AMH ELISA kit. Lesser AMH values have been reported by [33] with a range of AMH as 0.10 - 0.41 ng/mL in intact female dogs and ≤ 0.09 ng/mL in spayed dogs 10 days post operation using human based AMH ELISA kits. have reported mean AMH level of 0.30 ± 0.14 ng/mL in intact female dogs using human specific sandwich ELISA.[47] have reported a range of AMH level of 0.19 - 1.45 ng/mL and 0.01 - 0.06 ng/mL, respectively in intact female and spayed dogs 10 days post operation using canine specific kit by chemiluminescence immunoassay

The variation in the reported AMH values might be due to the type of kits used for the quantification, assay/quantitative technique used, unavailability of standardized kits in the market, and time of sample collection, particularly in females [48]. The AMH level is usually high during the proestrus and estrus stages of the cycle; hence, sampling for AMH estimation should be in those periods because its level gradually decreases from diestrus and reaches the basal level. Serum AMH concentrations have been observed to rise during late anestrus, peaking up to six days prior to ovulation, and then decrease significantly in the three days leading up to ovulation. (Walter *et al.*, 2019) [47].

The mean level of AMH in cryptorchid dogs in the present study was estimated to be 24.90 ± 0.79 ng/mL and is in accordance with the AMH value (> 23 ng/mL) reported by [35] using canine specific kits by chemiluminance assay [45] have reported lower median value of 1.61ng/mL (range 0.85 - 5.96) using human based AMH ELISA kits. A higher mean AMH of 54.98 ± 30.07 µg/L was reported by Hornakova *et al.* (2017) using human AMH kits by ECLIA. The difference in the reported AMH values might be attributable to the type of kits used for AMH estimation, quantitative technique used, and the unavailability of standardized kits in the market [48].

The mean AMH level of 3.20 ± 1.54 ng/mL in dogs with ORS was estimated in the present study. Concurrent mean AMH value of 4.40 ± 1.09 ng/mL has been reported by [49] using canine specific AMH ELISA kits. [33] have reported lower AMH value 0.18 and 0.19 ng/mL in two ORS cases using human based AMH kits. The reported variation in AMH values by different authors might be attributable to the type of kits used for AMH estimation, the quantitative technique used, and the unavailability of standardized kits in the market. Furthermore, the amount of ovarian tissue left in the body at the time of ovariectomy, subsequent tissue revascularization, amount of ovarian tissue developed, and time of sampling would influence the AMH level (Yilmaz *et al.*, 2015; Walter *et al.*, 2019) [47,49]. In this study, serum AMH concentrations were independent of the ovarian cycle in female dogs. However, ovarian hormones may affect the results of other diagnostic procedures including vaginal cytology, ultrasonography, and hormone stimulation tests (Wallace, 1991) [26]. This suggests a significant benefit of measuring serum AMH concentration for the diagnosis of ORS in female dogs.

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Based on the results (Figure 5), it was concluded that the serum AMH level of 0.26 ± 0.02 ng/mL in spayed, 5.14 ± 0.54 ng/mL in intact bitches, 16.20 ± 1.44 ng/mL in intact male dogs and 0.58 ± 0.05 ng/mL in castrated dogs can be considered as cut-off values [36]. Therefore, serum AMH measurement is the most reliable tool for ascertaining gonadal status in dogs of both sexes, even in the diagnosis of ORS and cryptorchidism (Figure 6). Diagnostic methods, such as history and clinical signs, are subjective, physical examination requires technical expertise, and ultrasonography is expensive, technically demanding, and time-consuming.

Table 1. Relative efficacy of different diagnostic methods for gonadectomy status in dogs.

Group	Gender	History & clinical signs	Physical examination	Trans- abdominal ultrasonography	AMH estimation
GROUP I (n=10) Gonadectomised	Male	5/5 (100%)	3/5 (60%)	4/5 (80%)	5/5 (100%)
	Female	5/5 (100%)	1/5 (20%)	0/5 (0%)	5/5 (100%)
GROUP II (n=16) Intact dogs	Male	8/8 (100%)	8/8 (100%)	8/8 (100%)	8/8 (100%)
	Female	8/8 (100%)	4/8 (50%)	2/8 (25%)	8/8 (100%)
GROUP III (n=3) ORS	Female	3/3 (100%)	2/3 (66.6%)	1/3 (33.3%)	3/3 (100%)
GROUP IV (n=8) Cryptorchid	Male	5/8 (62.5%)	2/8 (25%)	3/8 (37.5%)	8/8 (100%)

Note: The values in the parentheses indicate percentage

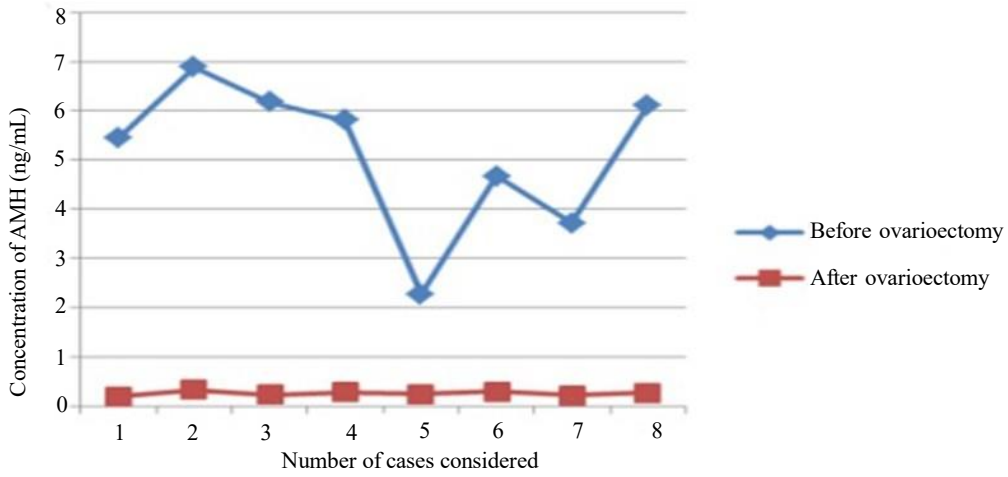

Figure 4. Concentration of AMH (ng/mL) in female dogs before and 10 days after ovariectomy.

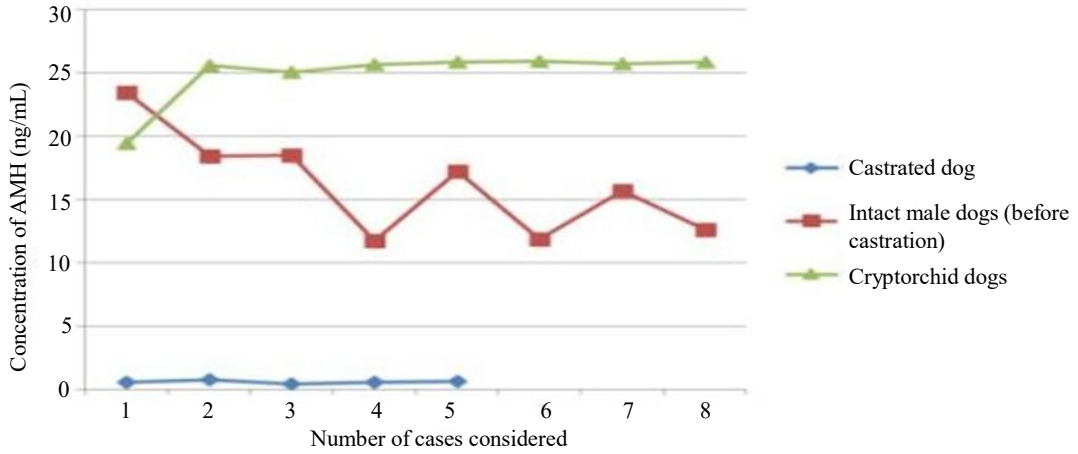

Figure 5. Concentration of AMH (ng/mL) in intact male (n=8), castrated (n=5) and cryptorchid (n=8) dogs.

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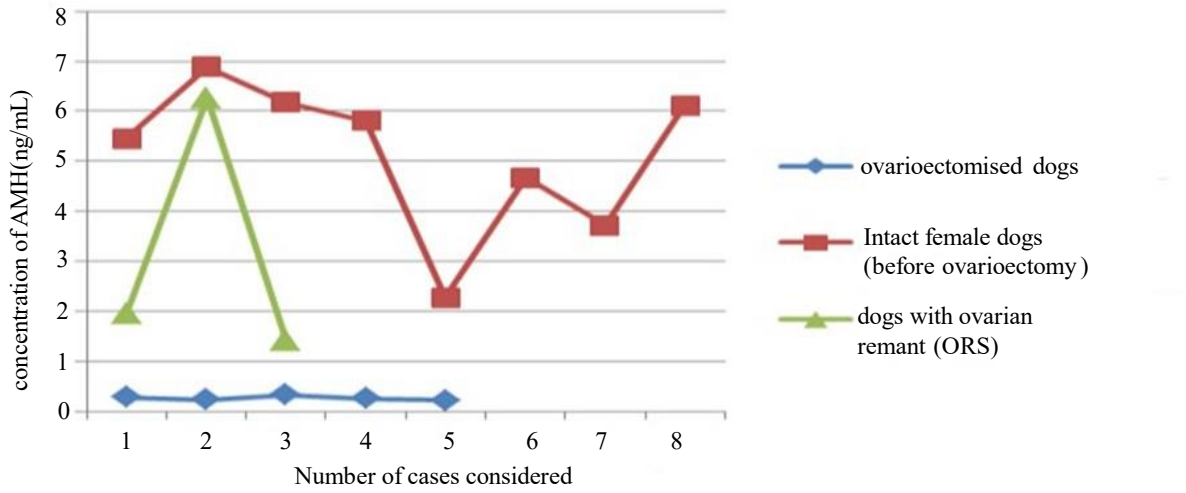

Figure 6. Concentration of AMH (ng/mL) in intact female (n=8), ovariectomised (n=5) and dogs with ORS (n=3)

Table 2. Overall relative accuracy (percentage) of various diagnostic methods for gonadectomy status in male and female dogs.

No. of dogs	History and clinical Signs		Physical examination		Ultrasonograp hy		AMH assay	
	Intact	***Neutered***	***Intact***	***Neutered***	***Inta ct***	***Neuter ed***	***Inta ct***	***Neuter ed***
Male (n=21)	13	5	10	3	11	4	16	5
Accuracy (%)	**85.71**		**61.90**		**71.42**		**100**	
Female (n=16)	11	5	6	1	3	0	11	5
Accuracy (%)	**100**		**43.75**		**18.75**		**100**	

CONCLUSION

This study aimed to evaluate various diagnostic methods for determining gonadal status in dogs, focusing on intact, cryptorchid, spayed, and castrated cases. History and clinical signs provided valuable insights, particularly when male dogs exhibited masculine behaviors, such as urine marking or mounting, and female dogs displayed signs of proestrus. However, owner-reported histories are not always reliable, especially for cryptorchid males and spayed females. Physical examinations helped confirm the presence of intact gonads in some cases, but factors such as obesity, lack of opportunity for male behavioral observation, and diversity in physical appearance among breeds. For females, the accuracy of physical examination is limited, especially in bitches with vulvar atrophy after ovariohysterectomy. Ultrasonography has emerged as a powerful non-invasive tool, demonstrating high sensitivity in locating retained testes and detecting ovarian remnants, but its effectiveness varies with operator skill, stage of the reproductive cycle, and tissue visibility. It was particularly useful in detecting cryptorchidism and assessing ovarian activity, although its accuracy in female dogs was lower than that in male dogs.

Serum Anti-Müllerian Hormone (AMH) measurement is the most reliable and objective diagnostic method. Distinct AMH levels were observed in the intact, spayed, castrated, cryptorchid, and ovarian remnant syndrome (ORS) cases. For instance, high AMH levels were observed in intact male dogs and cryptorchids, whereas low levels were indicative of spayed or castrated animals. The study concluded that AMH testing provides a consistent and accurate measure for determining gonadal status in both male and female dogs, surpassing other reliable diagnostic techniques, especially for diagnosing ORS and cryptorchidism. Therefore, it is recommended as the preferred diagnostic method by veterinarians.

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