# Anti-Müllerian hormone as an indicator of hemi-castrated unilateral cryptorchid horses

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*Anti-Müllerian hormone (AMH), a glycoprotein secreted from the fetal testis, is responsible for regression of the Müllerian duct in the male fetus. The aim of this study was to evaluate the usefulness of serum AMH as a biomarker for diagnosis of cryptorchidism in horses. Serum AMH concentrations were measured in intact stallions, hemi-castrated unilateral cryptorchid stallions, and geldings. In addition, expression of AMH was characterized in cryptorchid testes by immunohistochemistry. Serum AMH was detected in intact stallions (n=11, 13.3 ± 1.8 ng/ml) and in hemi-castrated cryptorchid stallions (n=8, 17.6 ± 3.0 ng/ml), but not in geldings (n=6, all data were below the limit of detection). Immunolabeling for AMH was detected in Sertoli cells of undescended testes from cryptorchid horses as well as those of normal testes. Our findings indicate that the cryptorchid testis after hemi-castration secretes AMH and that serum AMH concentrations may be a useful biomarker for diagnosis of equine cryptorchidism.*

Cryptorchidism, the failure of one or both testes to descend normally, is most frequently found in humans, pigs, and horses, although it does occur in many other mammalian species [7, 21]. The percentage of foals with cryptorchidism is estimated to be 5–8% (males), and most cases are unilateral cryptorchidism [3]. Horses with unilateral cryptorchidism are usually fertile, but bilateral cryptorchids are sterile. The interstitial (Leydig) cells, which produce testosterone, are not as heat sensitive as the cells of the seminiferous epithelium. Thus, although bilateral cryptorchid horses do not produce viable sperm, they usually exhibit masculine behavior [3]. In some cases, unilaterally cryptorchid horses have their single normally descended testis removed by incomplete castration and are erroneously considered geldings. These horses display stallion-like behavior, which is a frequent cause of blood hormone measurement to evaluate horses for retained testes.

The diagnosis of cryptorchidism can be complicated if a single normally descended testis has been removed [3]. A complete history, including behavior and previous surgery, is essential to make a correct diagnosis of cryptorchidism [17]. However, many horses have incomplete histories. Behavioral history is particularly important because masculine behavior is suggestive of a retained testis [17]. Strategies for diagnosis include palpation, ultrasound, or measurement of serum hormone levels. Some practitioners conduct rectal examinations on stallions to determine the testis location; however, the usefulness of rectal palpation to determine the location of a retained testis is controversial. Only some authors have found this test reliable [3]. In a previous study, preoperative diagnosis by rectal palpation of the vaginal rings was 87% accurate [19]. In another report, only two of 11 retained testes could be palpated rectally or

inguinally, whereas 100% of testes were found via ultrasonography [11].

Basal testosterone levels have been associated with up to a 14% error when attempting to distinguish geldings from cryptorchid horses [17]. Furthermore, serum conjugated estrogen concentrations have been reported to be 96% accurate for detecting cryptorchidism [4]. The human chorionic gonadotropin (hCG) stimulation test, which is the standard test for cryptorchidism, is reported to be 94.6% accurate for detecting the presence of testicular tissue [9]. However, this method is troublesome because it involves collecting multiple blood samples over 2 days and generates concern regarding the administration of a testosterone-inducing agent into racing or athletic horses. In addition, it is important to understand the specific reference range of individual laboratories when interpreting hormone assay results [17].

Anti-Müllerian hormone (AMH) is a homodimeric disulfide-linked glycoprotein of the transforming growth factor-β superfamily. It is secreted early in the fetal life of male animals by the Sertoli cells, where it induces regression of the Müllerian ducts [13]. Secretion of human AMH persists after birth [12, 18]. Postnatal secretion of human AMH is characterized by its high concentrations during the prepubertal period followed by a significant decrease at the onset of puberty [1, 5, 14]. Studies have described strong AMH expression in the Sertoli cells of the fetal equine testis, decreased expression in the prepubertal testis, and faint immunoexpression in the adult stallion testis [2, 6]. Serum AMH concentrations are higher in prepubertal colts than in colts post puberty [8]. Serum AMH concentrations in cryptorchid stallions are higher than those in intact stallions [8]. In contrast, serum AMH concentrations in geldings are at or below the limit detection of existing assays [8]. AMH is better known for its early ability to distinguish cryptorchidism from anorchia in prepubertal boys. Because AMH is a Sertoli cell-derived product, measurable amounts of AMH in blood are indicative of the presence of testicular tissue [10, 15, 16].

The objective of this study was to confirm the AMH secretion in the undescended testis in hemi-castrated unilaterally cryptorchid horses, in which the normal testis had previously been removed, by measuring serum AMH concentrations and performing immunohistochemistry and to discuss the usefulness of the AMH concentration as a diagnostic marker for equine cryptorchidism.

## Materials and Methods

*Animals*

Intact Thoroughbred stallions (n=11, 15.4 ± 1.9 years of age), Thoroughbred geldings (n=6, 11.2 ± 2.0 years of age), and hemi-castrated unilaterally cryptorchid horses with a history of surgical castration and no scrotal testis (four Thoroughbreds, two ponies, one quarter horse and one Westfalen, 6.3 ± 1.1 years of age) were used for examination of the general AMH concentration in serum. In addition, other intact Thoroughbred stallions (n=4, 2.3 ± 0.3 years of age) were used to investigate for changes in the serum AMH levels after surgical castration (castrated stallions). All horses were stabled on Hokkaido and Honsyu, Japan, and fed a balanced feed two or three times a day in their stables. This study was approved by the Animal Care and Use Committee at Hidaka Training and Research Center.

*Blood sampling*

The blood samples of intact stallions and geldings were collected in April. On the other hand, those for cryptorchid horses were collected in February, March, April, July, and August, and those for castrated stallions were collected in May and October respectively. The samples for castrated stallions were collected daily before and 2 weeks after surgical castration. Jugular venous blood samples were collected into vacuum tubes. Serum was decanted after centrifugation at 1,880 × g for 10 min at 4°C and stored at −20°C until use for the AMH immunoassay.

*Serum AMH assays*

Serum AMH concentrations were measured using a commercially available heterologous sandwich enzyme-linked immunoassay (AMH Gen II ELISA, #A73818, Beckman Coulter, Inc., Brea, CA, U.S.A.) according to the manufacturer's instructions. Serially diluted serums from two intact stallions were assayed to compare parallelism with bovine AMH-purified standard regents.

Briefly, 20 $\mu l$ of standards containing various AMH concentrations, controls, and samples were incubated in an anti-AMH antibody-coated microtiter plate. Samples with high AMH concentrations were diluted (1:5) with standard buffer to keep the assay results within the standard curve. After incubation and washing, anti-AMH biotin conjugate was added to each well. After a second incubation and washing step, streptavidin-horseradish peroxidase was added to each well. After a third incubation and washing step, a tetramethylbenzidine substrate was added to the wells, followed by an acidic stop solution. The degree of enzymatic turnover of the substrate was determined by dual-wavelength absorbance measurements at 450 and 615 nm using a microplate spectrophotometer (Multiskan Go, Thermo Fisher Scientific, Inc., Waltham, MA, U.S.A.). The lowest limit and intra- and interassay coefficients of variation for this assay were 0.16 ng/m$l$, <5.4%, and <5.6%, respectively. Values below the lowest limit, 0.16 ng/m$l$, were given a numerical value of 0 ng/m$l$ in this study.

*Immunohistochemistry*

Normal testes were obtained at surgical castration from a two-year-old Thoroughbred, and an undescended testis was obtained surgically from the abdominal cavity in a four-year-old pony. Testes were fixed in 4% paraformaldehyde, dehydrated through a graded ethanol and xylene series, embedded in paraffin, and sectioned at 4 $\mu$m for immunohistochemistry (IHC). The immunohistochemical procedure for detection of AMH expression was performed by the avidin-biotin-peroxidase complex method, as described previously [6]. After ABC staining, each slide was counterstained with hematoxylin. The primary antibody used was goat polyclonal anti-human AMH (1:500; sc-6886, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). The specificity of the primary antibody was assessed by incubating it with a blocking peptide (sc-6886P, Santa Cruz Biotechnology). The AMH antibody was mixed (1:5; w:w) with the peptide overnight at 4°C and then used for IHC.

*Statistical analysis*

A one-way analysis of variance was performed to compare AMH concentrations among intact stallions, cryptorchid stallions, and geldings. Tukey's HSD test was used to identify significant differences between individual means. Dunnett's test was also used to compare the serum AMH concentration before and after surgical castration. A *P*-value of <0.05 was considered significant. All statistical analyses were performed using the JMP software (SAS Institute Japan Inc., Tokyo, Japan). Data are presented as the mean ± standard error (SEM).

## Results

*Validation of the AMH assay for stallions*

The curves of serially diluted serum from two intact stallions were parallel to and within the range of the AMH standard curve (Fig. 1).

*Serum AMH concentrations in intact stallions, geldings, and hemi-castrated unilateral cryptorchid horses*

The serum AMH concentrations of the intact stallions and hemi-castrated unilateral cryptorchid horses were 13.3 ± 1.8 ng/m*l* (range, 1.7–21.9 ng/m*l*) and 17.6 ± 3.0 ng/m*l* (range, 3.1–28.2 ng/m*l*), respectively, and those of the geldings were below the limit of detection of the assay (Fig. 2). The serum AMH concentrations of the geldings were significantly lower than those in intact stallions and cryptorchid horses. The difference in the serum AMH concentration between intact stallions and hemi-castrated unilateral cryptorchid horses was not significant.
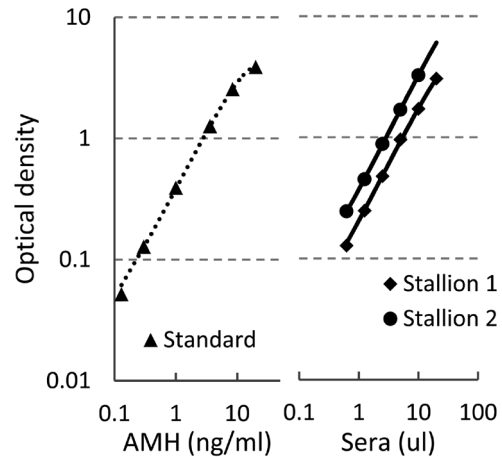


**Fig. 1.** Parallelism plot for the anti-Müllerian hormone (AMH) assay. The curves of the serially diluted serum samples from two intact stallions are parallel to the standard curve.
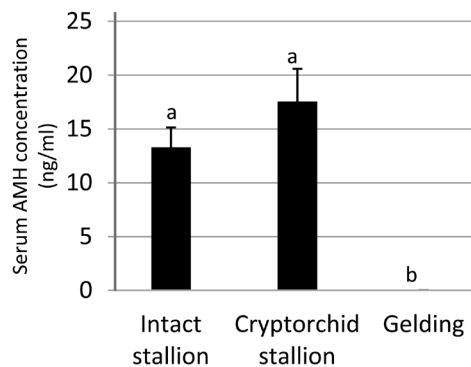


**Fig. 2.** Serum anti-Müllerian hormone (AMH) concentrations in intact stallions (n=11), cryptorchid stallions (n=8), and geldings (n=6). Different letters above the bars represent significant differences (*P*<0.05). Data are expressed as the mean ± standard error.

*Changes in serum AMH concentrations before and after castration*

The serum AMH concentrations before castration in four stallions were 8.7, 9.1, 11.3, and 27.0 ng/m*l*, respectively. The concentrations decreased after surgery, and were <1 ng/m*l* 8–10 days after surgery. The concentrations at 1 day after surgery were significantly lower than before castration. The biological half-life of AMH was about 2.5 days (Fig. 3).

*Immunohistochemical expression of AMH*

In cryptorchid testes, the layers of spermatogenic cells were thinner, seminiferous tubule diameters were smaller, and interstitial spaces were broader than those in normal testes. Immunohistochemical expression of AMH was observed in Sertoli cells but not in germ cells in normal and
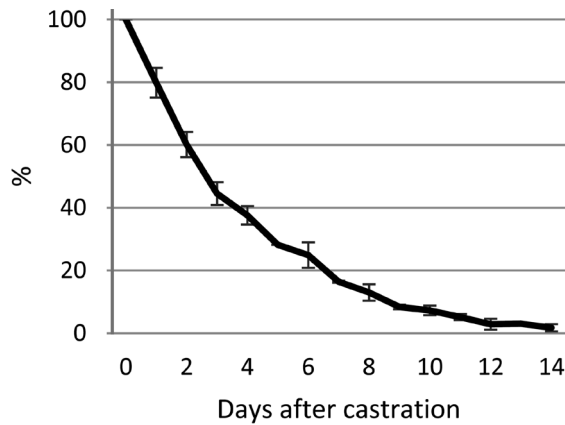
**Fig. 3.** Changes in serum anti-Müllerian hormone (AMH) levels after castration in stallions (n=4). The serum AMH concentrations before castration were considered to be 100%, and the values were 8.71, 9.12, 11.33, and 27.00 ng/m*l* respectively. The biological half-life of AMH was about 2.5 days. Data are expressed as the mean ± standard error.

cryptorchid testes. AMH immunolabeling was more intense in the intact testis than in the cryptorchid testis (Fig. 4A and 4B).

## Discussion

In the present study, the usefulness of measuring the serum AMH concentration as a marker for cryptorchidism using a commercial AMH ELISA kit was investigated. The Active AMH-ELISA (Diagnostic Systems Laboratories [DSL], Webster, TX, U.S.A.) has been reported to be useful for horses [8]. The usefulness of the AMH Gen II ELISA assay for horses, which uses DSL antibodies but different standardized calibrators, was evaluated in this study. In a previous study for women, there was good agreement between the DSL assay and the AMH Gen II assay, but the values from the AMH Gen II assay were approximately 40% higher than those in the human DSL assay [20].

Serum AMH was detected in intact stallions but not in geldings, as reported previously [8]. Serum AMH was also detected in bilateral and unilateral cryptorchid horses in a previous report [8]. In our results, AMH was detected in incomplete castrated unilateral cryptorchid horses. This suggested that the undescended testis also produces AMH. The AMH concentrations in cryptorchid horses are higher than those in intact horses [8]. In our study, the serum AMH concentrations in hemi-castrated unilateral cryptorchid horses tended to be higher than those in intact horses, but no significant difference was observed. Claes *et al.* [8] showed that the serum AMH concentrations are about 15 ng/m*l* in intact horses, 33 ng/m*l* in cryptorchid stallions, and below the limit of detection in geldings when measured by the DSL assay. Our results were lower than those of the DSL assay. This difference might depend on age and the presence of another testis. The serum AMH concentrations were higher in prepuberty than in post puberty [8]. The intact stallions we used were comparatively older ($15.4 \pm 1.9$ years of age). The hemi-castrated horses we used certainly lacked another testis. On the other hand, this information was not described in detail in the previous report [8].

The present findings concerning the immunohistochemical expression and localization of AMH in the cryptorchid testis were similar to those of a previous study [6], indicating
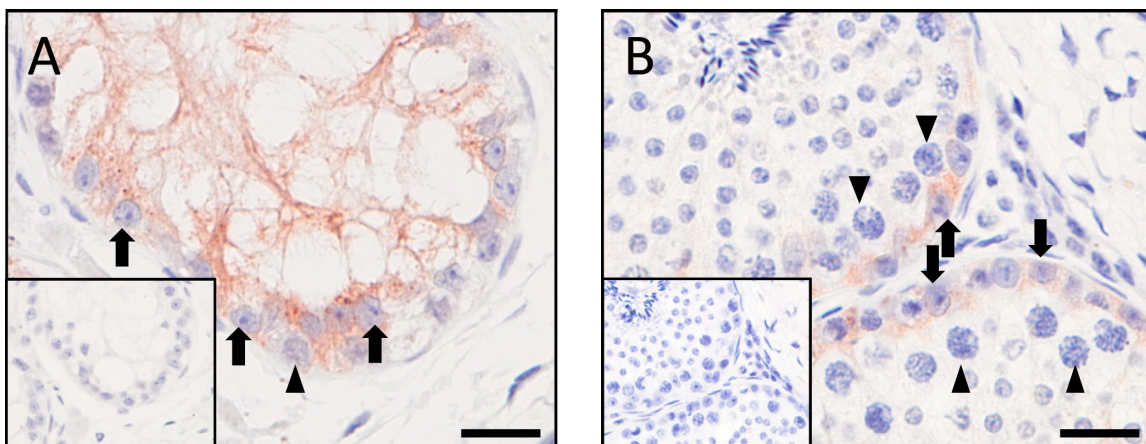


**Fig. 4.** Immunohistochemical detection of anti-Müllerian hormone (AMH) in equine cryptorchid (A) and normal testes (B). AMH immunolabeling was detectable in Sertoli cells (arrows) but absent in spermatocytes (arrowheads). There are fewer spermatocytes in cryptorchid testes. The inset shows negative staining with a blocking peptide. Scale bars=20 $\mu$m.

that AMH is produced in Sertoli cells of the undescended testis in hemi-castrated unilaterally cryptorchid horses. Also, our results showed lower immunostaining of AMH in the cryptorchid testis than in the intact testis. However, this decrease is not always attributable to cryptorchidism, because AMH immunostaining in the cryptorchid testis shows a reduction in intensity associated with age [6], and the ages of the horses in this study differed (the intact horse was 2 years old and cryptorchid horse was 4 years old). AMH expression in the normal testis is known to decrease with progression from the prepubertal to postpubertal to adult stages of testicular development in male horses [2]. The physiological significance of AMH secreted from the male gonad after birth remains an open question.

From our present study, it is clear that the testis is a major source of AMH secreted into the blood circulation in stallions because serum AMH was not detected two weeks after castration. To evaluate male horses for the presence of testes, measurement of circulating AMH levels in a blood sample is much more certain than measurement of steroid hormones in a single sample. Measurement of serum AMH concentrations was useful for identifying cryptorchid horses, although we did not compare this method with traditional diagnostic methods in this study.

In conclusion, we demonstrated AMH secretion in the undescended testis and the usefulness of the serum AMH concentration as a diagnostic biomarker for hemi-castrated unilaterally cryptorchid horses.

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