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Detection of AMH protein in bovine serum and production of polyclonal antibodies for fertility diagnostic applications

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Abstract

This study aimed to isolate and characterize Anti-Müllerian Hormone (AMH) protein from Peranakan Ongole (PO) cows and to produce polyclonal antibodies for potential reproductive biomarker applications. Nine PO cows were selected based on ultrasonographic and rectal palpation examinations, which confirmed active follicular development. Serum samples were collected and analyzed for total protein concentration, ranging from 55.73 to 70.23 mg/mL. SDS-PAGE analysis revealed varied protein profiles, with 3 to 10 bands observed per sample, indicating physiological diversity among the subjects. AMH protein was isolated using electroelution following SDS-PAGE separation. Western blot results confirmed that the immunogenic AMH protein was detected at a molecular weight of 60–70 kDa, aligning with the expected size for bovine AMH. The purified protein was used for immunization in rabbits to produce polyclonal antibodies. The successful detection and specificity of the anti-AMH antibodies support their potential use in reproductive physiology studies and the development of immunodiagnostic tools.

Keywords: Anti-Müllerian Hormone; PO cows; SDS-PAGE; Western blot; Antibody production; Reproductive biomarker

1. Introduction

Reproductive efficiency is a cornerstone of sustainable livestock production, particularly in the dairy and beef industries, where fertility directly impacts productivity and profitability. In recent decades, significant attention has been given to molecular biomarkers that can serve as early predictors of reproductive potential in livestock. Among these, Anti-Müllerian Hormone (AMH) has emerged as one of the most reliable indicators of ovarian follicular reserve and functional fertility in female animals, including cattle. AMH is a dimeric glycoprotein belonging to the Transforming Growth Factor-beta (TGF-β) superfamily, and is primarily secreted by granulosa cells of small growing ovarian follicles (Visser et al., 2006; Monniaux et al., 2013).

In bovines, serum concentrations of AMH have been positively correlated with the antral follicle count (AFC), which reflects the pool of recruitable follicles available in the ovary. This makes AMH a powerful tool for predicting ovarian response to hormonal stimulation protocols, such as those used in superovulation or in vitro embryo production (Rico et al., 2009; Mossa et al., 2012). Furthermore, studies have demonstrated that AMH levels are consistent within an individual across estrous cycles, making it a stable and non-invasive endocrine marker for fertility assessment (Ireland et al., 2011).

Despite its promising potential, the application of AMH as a routine fertility biomarker in veterinary practice is still limited, largely due to the lack of species-specific, cost-effective, and accessible diagnostic kits for AMH detection in cattle. While commercial ELISA kits exist, many are developed for human use and may not exhibit optimal sensitivity and specificity when applied to bovine samples. To bridge this gap, the development of polyclonal antibodies specifically targeting bovine AMH is critical. Polyclonal antibodies, which are produced by immunizing host animals such as rabbits with purified antigen, offer the advantage of recognizing multiple epitopes on the AMH molecule, thus enhancing detection sensitivity and robustness in various immunoassays (Abcam, 2023).

One of the key steps in the development of AMH-based diagnostics is the isolation and characterization of the AMH protein from bovine serum. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) allows for separation of proteins based on molecular weight, and can be used to estimate the presence of AMH. Typically, the full-length AMH precursor has a molecular weight of approximately 140 kDa, which is cleaved into a biologically active C-terminal fragment (~25 kDa) and a pro-region (~110–120 kDa). However, in Western blot assays, the AMH protein in bovine serum often appears as bands between 60–70 kDa, likely representing processed or partially glycosylated forms (Kumar et al., 2023).

The immunogenic AMH protein isolated from serum can then be used to generate polyclonal antibodies in rabbits. The resulting antibodies can be harvested from serum and characterized for their specificity via immunoblotting and other immunoassays. Once validated, these polyclonal antibodies can be incorporated into prototype diagnostic kits, such as indirect ELISAs, for the detection of endogenous AMH levels in cattle serum. These kits would represent a significant advancement in the field of veterinary reproduction, offering a practical and cost-effective tool for farmers, veterinarians, and breeding programs to assess female fertility potential and improve reproductive decision-making.

This study was designed to: (1) isolate AMH protein from bovine serum using SDS-PAGE, (2) identify the immunogenic AMH band through Western blot analysis using antisera, and (3) produce and validate polyclonal antibodies against bovine AMH in rabbits. The long-term goal is to support the development of a diagnostic kit for fertility monitoring in cattle, enhancing reproductive efficiency and supporting genetic selection programs in the livestock sector.

2. Material and methods

2.1. Study Design and Experimental Animals

This experimental study aimed to isolate Anti-Müllerian Hormone (AMH) protein from cow serum and produce polyclonal antibodies against AMH. A total of 20 Peranakan Ongole (PO) cows were used, divided into two groups based on physiological status: Group 1: 10 cows <60 days postpartum, Group 2: 10 cows >60 days postpartum. Body weight: approximately 250–300 kg. Body Condition Score (BCS): 2.5–3 (scale 1–5). Ultrasonography (USG) and rectal palpation were performed to assess reproductive status prior to blood collection.

2.2. Equipment and Materials

2.2.1. Equipment

Syringes and needles (10 mL), Plain vacutainer tubes, Centrifuge, Micropipettes and sterile tips, Nanodrop spectrophotometer, SDS-PAGE system (gel casting tray and electrophoresis unit), Electrophoresis power supply, Pre-stained protein ladder, Electroelution system, Western blot apparatus (transfer unit, PVDF/NC membranes), Incubator shaker, Vortex mixer, Water bath, Blot detection imaging system, Laboratory animal cages (for rabbits)

2.2.2. Materials

Cow blood serum, PBS buffer (Phosphate Buffered Saline), Laemmli buffer Acrylamide gel (30% acrylamide/bis-acrylamide), SDS (Sodium Dodecyl Sulfate), Tris-HCl buffer, Glycine, TEMED and APS (for gel polymerization), Complete and Incomplete Freund's Adjuvant, Secondary antibody (e.g., anti-rabbit HRP-conjugated), Detection substrates (e.g.,

DAB, ECL), Blocking reagent (e.g., skim milk or BSA), Transfer buffer (Tris-Glycine-Methanol), Male New Zealand White rabbits (7 animals, 2–3 kg)

2.3. Serum Isolation and AMH Protein Characterization

Blood was collected from the jugular vein, and serum was isolated by centrifugation. The AMH protein in serum was characterized using SDS-PAGE: Acrylamide gel was prepared. Serum was mixed with Laemmli buffer in a 1:3 ratio. A pre-stained protein ladder was used to identify the target AMH molecular weight. Electrophoresis was run at 90–120 volts for 90 minutes.

2.4. AMH Protein Isolation via Electroelution

Following SDS-PAGE: Target AMH bands were excised from the gel. Gel slices were placed into the electroelution chamber. Electroelution was run at 90–120 volts for 120 minutes. Isolated AMH protein was collected and stored at low temperature until further use.

2.5. Polyclonal Antibody Production in Rabbits

A total of 7 male New Zealand White rabbits (2–3 kg) were used. The concentration of isolated AMH protein was measured using a Nanodrop spectrophotometer. The protein was administered subcutaneously (SC): First injection: mixed with Complete Freund's Adjuvant (CFA). Subsequent injections: mixed with Incomplete Freund's Adjuvant (IFA). Immunization was performed 6 times at 2-week intervals.

Blood samples were collected: Before the second injection (baseline), Two weeks after the final injection, Rabbits were sacrificed for final blood collection. Serum was isolated for antibody titer analysis.

2.6. Western Blotting

Western blotting was performed to confirm the presence and molecular weight of AMH protein and to evaluate antibody specificity: Sample preparation (antigen). Protein separation using SDS-PAGE. Transfer of protein to PVDF/NC membrane. Blocking of non-specific binding sites. Incubation with primary antibody (rabbit serum), followed by secondary antibody (e.g., anti-rabbit HRP). Detection using enzymatic or chemiluminescent substrate (e.g., DAB or ECL).

3. Results and discussion

3.1. Results of Ultrasonography (USG) and Rectal Palpation

Reproductive examinations of the cows were conducted using ultrasonography (USG) and rectal palpation to assess the physiological status of the reproductive organs, including uterus and ovaries, and to confirm the absence of pregnancy. Results of ultrasonography (USG) and rectal palpation can be seen in Table 1.

Table 1 Results of ultrasonography (USG) and rectal palpation

Number	Earteg-Sample Code	Examination	Average Ovarian Diameter		Evidence
			Left	Right	
1	Po 14/85-(A)	USG and palpation	2,71	2,15	There is follicle growth
2	Po 18/86-(B)	USG and palpation	1,15	1,56	There is follicle growth
3	Po 18/76 -(C)	USG and palpation	1,32	1,39	There is follicle growth
4	Po 18/29 -(D)	USG and palpation	2,78	2,51	There is follicle growth
5	Po 15/140 -(E)	USG and palpation	1,78	1,89	There is follicle growth
6	Po 18/24-(F)	USG and palpation	2,82	2,71	There is follicle growth
7	Po 19/17-(G)	USG and palpation	2,53	2,12	There is follicle growth
8	Po 13/109-(H)	USG and palpation	2,63	2,47	There is follicle growth
9	Po 19/37-(I)	USG and palpation	1,76	1.77	There is follicle growth

Ovarian examination of Peranakan Ongole (PO) cows through ultrasonography and rectal palpation revealed that all examined individuals exhibited normal ovarian activity. Each of the nine cows displayed evidence of follicular growth in either or both ovaries, suggesting that they were in the follicular phase or actively cycling. This finding is highly significant in the context of this study, particularly in relation to the production and isolation of Anti-Müllerian Hormone (AMH), as AMH expression is associated with the presence of growing follicles.

The recorded ovarian diameters varied among individuals. The left ovaries ranged from 1.15 cm to 2.82 cm, while the right ovaries ranged from 1.39 cm to 2.71 cm. Physiologically, ovarian diameters above 1.5 cm typically indicate the presence of dominant or actively developing follicles. These measurements therefore support the conclusion that all examined cows were experiencing normal follicular activity.

One particularly notable case was observed in the individual with sample code Po 18/24-(F), which showed the largest ovarian diameters—2.82 cm on the left and 2.71 cm on the right—indicating vigorous follicular development and excellent reproductive potential. Meanwhile, cows such as Po 18/86-(B) and Po 18/76-(C) exhibited smaller ovarian diameters, but still demonstrated follicular activity, and thus cannot be classified as having inactive ovaries.

The fact that all cows exhibited bilateral ovarian activity suggests a sufficient population of antral follicles. This is especially relevant for this research, as the presence of such follicles is closely linked to AMH secretion within the reproductive system. Consequently, the cows used in this study can be considered physiologically suitable as a source for AMH protein isolation and subsequent antibody production.

The protein profiles of nine different samples (PO 14/85-(A) to PO 19/37-(I)) were analyzed using SDS-PAGE, revealing diverse patterns in both the number of bands and their molecular weights (Table 1). The total number of protein bands ranged from 3 to 10, reflecting variability in protein complexity among samples. Samples PO 15/140-(E), PO 18/24-(F), PO 19/17-(G), PO 13/109-(H), and PO 19/37-(I) consistently showed the highest number of bands (10 bands each). This suggests a more complex proteome profile compared to samples like PO 18/86-(B) and PO 18/29-(D), which exhibited fewer bands (3 bands each). The variation in band number and intensity is likely indicative of differences in physiological status, protein expression levels, or sample processing and preparation techniques (García-Cañas et al., 2017). Total protein concentration of serum samples from PO cows

Tabel 2 Total protein concentration of serum samples from PO cows can be seen in Table 2

Number	Sample Code	Protein Level Mg/Ml
1	PO 14/85-(A)	62.03
2	PO 18/86-(B)	55.73
3	PO 18/76 -(C)	70.03
4	PO 18/29 -(D)	63.82
5	PO 15/140 -(E)	64.92
6	PO 18/24-(F)	59.96
7	PO 19/17-(G)	57.22
8	PO 13/109-(H)	70.23
9	PO 19/37-(I)	62.78

This dataset presents the total protein concentration (in mg/mL) measured from serum samples of nine individual Ongole (PO) cows, each identified with a specific sample code. The protein concentrations range from 55.73 mg/mL to 70.23 mg/mL. Sample PO 13/109-(H) exhibited the highest protein concentration at 70.23 mg/mL, followed closely by PO 18/76-(C) at 70.03 mg/mL, indicating a potentially higher level of protein expression or physiological activity in these animals. Conversely, the lowest protein concentration was observed in PO 18/86-(B) at 55.73 mg/mL. Other samples such as PO 19/17-(G) and PO 18/24-(F) also showed relatively lower protein levels at 57.22 mg/mL and 59.96 mg/mL, respectively. The variation in protein levels across the samples may be influenced by multiple factors including physiological status, reproductive stage, metabolic activity, or individual health condition. This protein concentration data is essential for downstream analyses such as SDS-PAGE, protein purification, or antibody production, where accurate quantification is critical for standardizing experimental input.

Table 3 Protein band number and molecular weights (kDa) per sample

No	Kode Sampel	Jumlah Band	Berat Molekul (kDa)
1	PO 14/85-(A)	4	177,64; 35,97; 31,55; 14,66
2	PO 18/86-(B)	3	200,75; 34,71; 29,85
3	PO 18/76-(C)	4	177,64; 34,71; 29,85; 16,50
4	PO 18/29-(D)	3	177,64; 34,71; 30,66
5	PO 15/140-(E)	10	177,64; 141,30; 104,06; 79,88; 52,50; 35,97; 31,55; 29,10; 17,66; 15,90
6	PO 18/24-(F)	10	200,75; 141,30; 104,06; 68,40; 49,57; 35,97; 32,51; 28,40; 17,08; 14,66
7	PO 19/17-(G)	10	200,75; 141,30; 94,86; 73,77; 46,95; 35,97; 32,51; 28,40; 17,08; 14,66
8	PO 13/109-(H)	10	177,64; 141,30; 104,06; 68,40; 46,95; 37,37; 32,51; 29,10; 17,66; 15,90
9	PO 19/37-(I)	10	200,75; 141,30; 104,06; 73,77; 49,57; 38,91; 34,71; 29,10; 18,21; 15,90

The presence of high molecular weight protein bands, particularly those above 100 kDa, was observed predominantly in samples with 10 bands. For instance, proteins at approximately 200 kDa, 177 kDa, 141 kDa, and 104 kDa were detected in most of these samples. High molecular weight proteins often correspond to structural proteins such as cytoskeletal components or protein complexes essential for cellular integrity and function (Kumar et al., 2019). These bands might also represent glycoproteins or membrane proteins, which tend to have higher molecular weights due to post-translational modifications (Walsh, 2014). The recurring appearance of bands near 177 kDa in the majority of samples (except PO 19/17-(G)) suggests a conserved protein possibly playing a crucial role across all samples. Differences in the intensity or absence of this band in some samples might reflect variations in protein abundance or possible proteolytic cleavage (Aebersold & Mann, 2016).

Bands in the medium molecular weight range (30–80 kDa) were commonly found across all samples. These included bands around 35–38 kDa, 29–32 kDa, and 46–49 kDa. Proteins within this range are frequently enzymes, signaling proteins, or transport proteins that perform various cellular functions (Brunak et al., 2017). The smaller proteins, particularly those below 30 kDa, appeared consistently across the samples with notable bands at approximately 15–18 kDa and 14–17 kDa. These low molecular weight bands might correspond to regulatory peptides, small enzymes, or proteolytic fragments resulting from protein degradation during sample preparation (Righetti, 2018).

The variation in the protein band profiles among the samples could be attributed to biological differences such as genetic variation, tissue type, physiological or pathological conditions, or environmental influences (Li et al., 2020). For instance, the samples exhibiting more complex profiles with additional protein bands might correspond to tissues with higher metabolic or structural activity, or samples exposed to different external stimuli. The consistent presence of certain molecular weight bands across all samples implies the expression of essential housekeeping proteins involved in fundamental cellular processes such as cytoskeletal maintenance, energy metabolism, or protein synthesis (Gstaiger & Aebersold, 2009). The detection of specific protein bands unique to certain samples may serve as biomarkers for physiological states or disease conditions and warrants further identification and characterization (Zhang et al., 2015).

These findings align with previous studies where protein banding patterns from SDS-PAGE analysis exhibited similar molecular weight ranges and band variability depending on the sample source and condition (Nguyen et al., 2018). Several reports have noted that protein profiles with multiple bands in the range of 10 to 200 kDa are typical in complex tissue extracts or biological fluids (Sun et al., 2016). Moreover, differences in protein profiles have been linked to changes in expression during development, disease progression, or in response to environmental stressors (Kang et al., 2019). The current data reinforce the importance of protein profiling for understanding biochemical and physiological diversity among biological samples.

While SDS-PAGE provides a valuable overview of protein complexity and molecular weights, it does not offer precise protein identification. To further elucidate the identity and function of these proteins, complementary techniques such as mass spectrometry or western blotting using specific antibodies are recommended (Aebersold & Mann, 2016). Additionally, quantitative analyses could provide insights into differential protein expression levels between samples. Future studies could also focus on correlating the observed protein profiles with functional assays or physiological data to better understand the biological relevance of these proteins in the studied context. The extract pumpkin seed contain

mineral Zn which can protect testicular damage due to exposure to heat, heavy metals, and fluoride. The results of spermatozoa from domestic rooster exposed to heat stress and treated with pumpkin seeds extract showed positive results both macroscopically and microscopically show in Table 1. It is caused by mineral Zn from Extract pumpkin seed allowing for the optimal spermatogenesis, good conditions are created from the seminiferous tubules to the epididymis that can maintain spermatozoa life optimally (Mustafa *et al.*, 2017). This is related to the function of hormones to maintain complementary genital organs which will produce seminal plasma for metabolism of spermatozoa and as a source of food substances for spermatozoa to live.
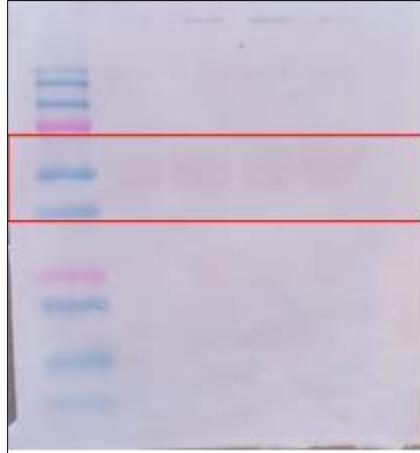

Figure 1 Westernblot results show that the immunogenic anti-AMH protein has a molecular weight of 60-70 kDa

The results of the Western blot analysis revealed that the immunogenic protein recognized by the anti-AMH (Anti-Müllerian Hormone) antibody appeared at a molecular weight of approximately 60–70 kDa. This indicates that the isolated and purified AMH protein corresponds to the expected molecular size of AMH in bovine species, consistent with previous reports. The appearance of a distinct band within this molecular weight range suggests that the purified AMH protein was immunogenic, capable of eliciting an immune response and specifically recognized by the polyclonal anti-AMH antibodies produced during the study. The presence of a clear single band also reflects the success of the protein purification process, including SDS-PAGE separation and electroelution, with minimal contamination from non-target proteins.

Biologically, AMH is a dimeric glycoprotein belonging to the TGF-β superfamily, and its molecular weight can vary depending on whether it is in the precursor (proAMH) or mature form. Therefore, the detected 60–70 kDa band may represent the active or oligomeric form of AMH that retains the specific epitopes necessary for antibody recognition. These findings confirm that the AMH protein isolation and polyclonal antibody production protocols used in this research were appropriate and effective. The successful detection of AMH protein through Western blot also provides critical validation of the antibody's specificity, supporting its potential application in future immunodiagnostic tools or reproductive physiology research.

4. Conclusion

This study successfully demonstrated that Peranakan Ongole (PO) cows used as experimental animals exhibited active ovarian follicular development as confirmed by ultrasonography and rectal palpation. Serum protein analysis revealed varying total protein concentrations ranging from 55.73 to 70.23 mg/mL, with SDS-PAGE profiling showing diverse protein band patterns, indicating physiological variability among the cows. Western blot analysis confirmed that the isolated Anti-Müllerian Hormone (AMH) protein was immunogenic, appearing at the expected molecular weight of 60–70 kDa. This supports the specificity of the polyclonal anti-AMH antibody produced. Overall, the isolation and identification of AMH protein and the subsequent production of specific antibodies were successful, providing a foundation for future applications in reproductive diagnostics and biomarker development.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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