Detection of Anti-Müllerian Hormone (*AMH*) mRNA in Serum and Ovarian Tissue of Local Indonesian Cattle Using EvaGreen-based RT–qPCR

Dicky Mohammad Dikman[1,2]*, Heni Puspitasari[3], Seagames Waluyo[4], Muhammad 'Ahdi Kurniawan[5,6], Supriyadi[7], Pudji Srianto[8], Epy Muhammad Luqman[9], Tri Wahyu Suprayogi[8], Sri Pantja Madyawati[10], Erma Safitri[8], Tita Damayanti Lestari[8]

[1]Doctoral Program of Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya 60115, Indonesia, [2]Beef Cattle Research Institute, Pasuruan 67184, East Java, Indonesia, [3]Research Group of Toxoplasma, Universitas Airlangga, Surabaya 60115, East Java, Indonesia, [4]Pt. Sciencewerke, Palmerah, West Jakarta City, Special Capital Region of Jakarta, [5]Master Program of Veterinary Science and Public Health, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya 60115, Indonesia, [6]Medical Biotechnology Research Group, Virtual Research Center for Bioinformatics and Biotechnology, Surabaya, Indonesia, [7]Founder and Scientific Researcher of Pos Tugas, Indonesia, [8]Department of Veterinary Reproduction, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya 60115, Indonesia, [9]Department of Veterinary Anatomy, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya 60115, Indonesia, [10]Department of Veterinary Sciences, Division of Veterinary Reproduction, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya 60115, Indonesia.
*Corresponding author: dickydikman@gmail.com

Abstract

Anti-Müllerian hormone (*AMH*) has been identified as a potential biomarker for assessing ovarian reserve and reproductive capacity in cattle, with high heritability and relatively stable expression. However, studies detecting *AMH* mRNA in bovine serum remain scarce due to the matrix's inherently low RNA yield, susceptibility to degradation, and potential presence of PCR inhibitors. This study provides an exploratory validation of *AMH* mRNA detection in serum — a challenging matrix compared with ovarian tissue — using EvaGreen-based reverse transcriptase–quantitative PCR (RT–qPCR). Specific primers for the *AMH* and *β-actin* genes were designed *in silico* and validated through melting curve analysis and linearity testing. The results showed high amplification efficiency (*AMH*: 100.2%, $R^2 = 0.994$; *β-actin*: 109.1%, $R^2 = 0.996$), with specific amplification of both targets. *AMH* detection in serum samples was successful in some samples, while the *β-actin* gene was consistently amplified as a reference gene. Despite the low RNA quality from serum and the presence of organic contaminants, the method demonstrated its feasibility for detecting *AMH* transcripts in a minimally invasive manner. Physiologically, *AMH* levels positively correlate with antral follicle count, superovulation success, and embryo quality, and are sensitive to heat stress and other environmental factors. These findings provide a foundational basis for developing molecular diagnostic approaches based on *AMH* gene expression in cattle reproductive management programs and support the future development of efficient, accurate, and context-specific biomolecular-assisted selection technologies for tropical livestock systems.

Keywords: Anti-Müllerian Hormone, gene expression, molecular biomarker, fertility, real-time PCR

Received: June 17, 2025 **Revised:** August 17, 2025 **Accepted:** September 17, 2025

INTRODUCTION

Reproductive success is a key factor in determining the efficiency and profitability of dairy farms. Delayed conception after the voluntary waiting period can lead to reduced milk production and reproductive efficiency (Vidal *et al*., 2024). Therefore, selection and management strategies that emphasize reproductive and productive potential are crucial, with an ideal target of first calving at around 24 months to reduce costs and accelerate the breeding cycle (Boulton *et al*., 2017). However, conventional reproductive parameters such as calving interval, postpartum gestation period, and number of inseminations have low heritability (<5%) and are highly influenced by management and environmental factors (Berry *et al*., 2014).

Consequently, alternative phenotypic traits that are more stable, easier to measure, have high heritability, and are less affected by external conditions are needed (Meier *et al*., 2021).

Anti-Müllerian Hormone (*AMH*) has been identified as a potential biomarker for assessing bovine fertility. Low *AMH* levels are associated with low post-estrus pregnancy rates and an increased risk of early miscarriage (Ribeiro *et al*., 2014). *AMH* has the advantage of high heritability (0.36–0.43) compared to conventional reproductive traits (0.01–0.13) (Nawaz *et al*., 2018; Alward *et al*., 2020), making it a promising indicator in genetic selection. Cows with high *AMH* levels are also known to have longer productive lives and more lactations (Koca *et al*., 2024a), reinforcing their role as an early marker of economic value and longevity in female cattle. Within the framework of sustainable livestock farming, fertility and longevity of female cattle are critical aspects (Kertz *et al*., 2023). However, infertility or subfertility remains a major challenge even when nutrition is adequate, with physiological and genetic factors being the dominant causes (Marrella and Biase, 2023). Therefore, early identification and accurate selection of cows with optimal reproductive potential are crucial to ensuring the dairy industry's sustainability (Kertz *et al*., 2023; Marrella and Biase, 2023).

AMH is a glycoprotein from the TGF-β superfamily that acts through the activation of a heteromeric receptor system (Mizuno *et al*., 2024). In cattle, *AMH* consists of 575 amino acids (Moolhuijsen and Visser, 2020), and circulating *AMH* levels are known as accurate endocrine indicators for assessing ovarian response to superovulation and the efficiency of transvaginal oocyte retrieval (Hirayama *et al*., 2017; Gobikrushanth *et al*., 2018). Therefore, *AMH* is considered a key marker in selecting cattle with high reproductive potential, particularly for identifying oocyte and embryo donors (Karl *et al*., 2022; Krause *et al*., 2022). *AMH* levels also positively correlate with antral follicle count (AFC), which is closely associated with fertility levels (Koyama *et al*., 2018). Although pre-antral follicles cannot be directly visualized, the total number of ovarian follicles strongly correlates with AFC (Feres *et al*., 2024). Given the association between AFC and the success of assisted reproductive technology (ART), *AMH* is considered a biomarker of oocyte and embryo quality in humans (La Marca *et al*., 2016) as well as in livestock such as cattle, sheep, poultry, and goats (McGrice *et al*., 2020; Krause *et al*., 2022).

Reverse transcriptase–quantitative PCR (RT–qPCR) has become the preferred molecular method for quantifying gene expression due to its sensitivity and precision. This method begins with the synthesis of complementary DNA (cDNA) from messenger RNA (mRNA) using reverse transcriptase, which then serves as a template in RT-qPCR amplification (Bustin and Nolan, 2020). This technique enables real-time monitoring of DNA amplification through fluorescence, with the cycle threshold (Ct) value serving as a quantitative marker for gene presence (Manurung and Sukohar, 2021). RT-qPCR has a wide range of applications, including gene mutation analysis, pathogen detection, identification of transgenic organisms, detection of single nucleotide polymorphisms, and analysis of *AMH* gene expression (Costella *et al*., 2018; Barra *et al*., 2019; Chhalliyil *et al*., 2020; Kurniawan *et al*., 2025). In the context of cattle reproduction, the detection of *AMH* mRNA in serum offers an earlier and potentially more sensitive method for evaluating reproductive potential (Koca *et al*., 2024b; Yildiz *et al*., 2024). This molecular approach can enhance reproductive selection strategies by correlating serum *AMH* mRNA levels with circulating *AMH* protein, thereby providing a practical and accurate tool for breeding management.

Despite its well-documented importance as a fertility biomarker, most studies in cattle focus on *AMH* protein concentrations in plasma or serum via ELISA. In contrast, *AMH* mRNA expression is primarily analyzed in ovarian tissue. There is a critical lack of research on detecting *AMH* mRNA directly from bovine serum a challenging biological matrix, due to its low RNA yield, susceptibility to degradation, and potential PCR inhibitors. This knowledge gap limits the development of minimally invasive, early-

detection molecular assays that could complement or replace protein-based methods for fertility prediction in cattle. Therefore, this study aims to detect and analyze *AMH* mRNA in bovine serum using RT-qPCR, evaluating its feasibility as an alternative, minimally invasive molecular tool for reproductive selection in cattle.

MATERIALS AND METHODS

Ethical Approval

This study received ethical clearance from the Animal Welfare Commission of Baltbangtan (KKHB), Agricultural Research and Development Agency of Indonesia (Approval No: Balitbangtan/Lolitsapi/Rm_Rd/07.01/2021).

Study Period and Location

The study was conducted between July and September 2021. Tissue and serum samples were aseptically collected from local Indonesian cattle at the Large Ruminant Agricultural Instrument Standardization Agency (BSIP), Grati, Pasuruan, East Java. All samples were immediately stored at –20 °C to preserve RNA integrity until further processing. Subsequent laboratory procedures, including total RNA extraction, complementary DNA (cDNA) synthesis, and analysis of AMH gene expression using real-time PCR (qPCR), were carried out at the Laboratory of the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, East Java.

Sample Collection and RNA Extraction

Total RNA was extracted from bovine ovarian tissue samples using the TIAM Genomic Extraction Kit (TIANGEN, China). Approximately 200 mg of tissue was homogenized in 1 mL of sterile double-distilled water (ddH_2O), followed by centrifugation at 12,000 rpm for 5 minutes in a 1.5 mL microcentrifuge tube. After discarding the supernatant, the resulting pellet was resuspended in 200 µL of GA buffer. The subsequent steps were carried out according to the manufacturer's protocol.

The quantity and purity of extracted RNA were evaluated using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, USA), measuring absorbance ratios at 260/280 and 260/230 nm. To assess RNA integrity and detect possible degradation, 1% agarose gel electrophoresis was conducted. Due to the unavailability of a Bioanalyzer system, RNA Integrity Number (RIN) could not be determined. Each RT-qPCR reaction was performed in technical triplicate for all samples to ensure measurement precision and reproducibility, by MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines.

Sample Preparation, RNA Extraction, and cDNA Synthesis

Serum samples (n = 4; 200 µL) from 1-year-old cattle and fresh tissue samples (n = 2; 200 mg) were used for total RNA extraction. The extraction was conducted using the Aurum Total RNA Mini Kit (Bio-Rad, CA, USA), following the manufacturer's protocol. For cDNA synthesis, 2 µL of the extracted RNA was used in a 10 µL reaction volume with the iScript™ cDNA Synthesis Kit (Bio-Rad), consisting of 2 µL reaction buffer, 0.5 µL reverse transcriptase enzyme, 2 µL RNA template, and 5.5 µL nuclease-free water. The thermal cycling conditions included incubation at 25 °C for 5 min (primer annealing), 48 °C for 20 min (reverse transcription), and 95 °C for 1 min (enzyme inactivation). The reactions were performed using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad).

Primer Design

Primer specificity was defined as the ability of a primer to align with the target gene sequence with greater than 80% identity. Forward and reverse primers were designed to amplify the coding sequence (CDS) regions of the *Anti-Müllerian Hormone* (*AMH*; GenBank accession no. M13151.1; Cate *et al*., 1986) and *β-actin* (GenBank accession no. AY141970.1; Suchyta *et al*., 2004) genes using the Primer-BLAST tool provided by the National Center for Biotechnology Information (NCBI). The designed primer pairs were evaluated *in silico*,

and melting curve analysis was performed to confirm amplification specificity, ensuring that only primer sets generating single-peak melt curves were selected. The finalized primer pairs yielded expected amplicon sizes of 217 base pairs (bp) for *AMH* and 103 bp for *β-actin*. All selected primers were synthesized by a commercial provider (insert supplier if known).

qPCR Optimization

All RT-qPCRs were conducted using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA). Each 10 µL reaction mixture consisted of 5 µL of EvaGreen Supermix (a DNA-binding dye-based RT-qPCR kit; Bio-Rad), 0.5 µL of forward primer (10 pmol), 0.5 µL of reverse primer (10 pmol), 3 µL of template DNA, and 1 µL of nuclease-free water. The thermal cycling protocol included an initial denaturation at 95 °C for 3 min, followed by 43 cycles of denaturation at 94 °C for 15 s and combined annealing/extension at 65–72 °C for 30 s. Before laboratory amplification, *in silico* melting curve analyses were conducted using the uMelt software, complemented by annealing temperature (Ta) optimization through gradient RT-qPCR. The melting curve analysis was performed from 70 °C to 95 °C, with fluorescence readings acquired every 0.5 °C per s to assess the specificity of the amplification. Once the optimal Ta was determined, reproducibility and reaction linearity assessments were performed to validate the assay.

qPCR Amplification

qPCR of *AMH* and *β-actin* gene expression was performed on cDNA from serum samples using the CFX96 Touch system (Bio-Rad). Each 10 µL RT-qPCR reaction mixture consisted of 5 µL EvaGreen Supermix (Bio-Rad), 0.5 µL of forward primer (10 pmol), 0.5 µL of reverse primer (10 pmol), 3 µL of cDNA template, and 1 µL of nuclease-free water. The thermal cycling protocol included an initial denaturation at 95 °C for 3 min, followed by 43 cycles of denaturation at 94 °C for 15 s, and annealing/extension at 65 °C for *β-actin* and 70 °C for *AMH*, each for 30 s. A melting curve analysis was performed from 70 °C to 95 °C, with fluorescence readings acquired every 0.5 °C per second to confirm amplification specificity.

Data Analysis

The expression level of the *AMH* gene was quantified using the comparative Ct (2^−ΔCt) method, with *β-actin* serving as the endogenous reference gene. For each sample, Ct values of both the target and reference genes were recorded. Only RT-qPCR reactions that generated a single, well-defined melting peak were considered valid and included in the analysis, while reactions showing multiple peaks or irregular fluorescence curves were excluded. Relative gene expression levels were calculated using the 2^−ΔCt formula and expressed as mean ± standard deviation (SD). Descriptive statistical analysis was performed to evaluate expression patterns of *AMH* mRNA in both serum and tissue samples, as well as to explore inter-individual variability and assess the reliability of detection across different sample types. Statistical evaluations of Ct values, amplification efficiency, and variability among technical replicates were conducted using Microsoft Excel 2019 and GraphPad Prism 9.0. Mean, standard deviation, and coefficient of variation (CV) were calculated to assess amplification consistency and reproducibility, with a descriptive significance threshold set at $p < 0.05$ for applicable parametric comparisons.

RESULTS AND DISCUSSION

***In silico* Analysis**

The resulting primers for *AMH* were 5′-CCTTGCTGAGGTTCCAGGAG-3′ (forward) and 5′-AGGGTAAGGGCTAACCCAGG-3′ (reverse), while those for *β-actin* were 5′-AGAGCAAGAGAGGCATCC-3′ (forward) and 5′-TCGTTGTAGAAGGTGTGGT-3′ (reverse). Comparative analyses of the target gene sequences were performed against the sequences from various species using the NCBI database to ensure primer specificity. The *AMH* primers amplified a 217 bp region (nt 683–880), and the *β-actin* primers targeted a 103 bp region (nt 214–314), as illustrated in Figure 1. As shown in Table

1, both primer sets exhibited 100% sequence identity with those from *Bos taurus*, *Bos indicus*, and *Bison bison*, and the *β-actin* primers showed a perfect match with *Bos mutus*. The *AMH* primers showed 97.5% identity with the *Bos mutus* sequence. In contrast, sequences from non-target species, such as *Equus caballus*, *Gallus gallus*, *Sus scrofa*, *Mus musculus*, and *Rattus norvegicus*, showed poor alignment or mismatches with the *AMH* primers, indicating potential non-specific amplification. The *β-actin* primers showed moderate-to-high similarity with the sequences in these species but remained less specific than their match with Bos. Both primer sets showed no significant alignment with non-mammalian organism sequences, such as *Oryza sativa* and *Zea mays* sequences, suggesting no risk of cross-amplification in plant species. These findings confirm the high specificity of the designed primer sets for detecting *AMH* gene expression in Bos, particularly for use in local Indonesian cattle.

Table 1. Comparative sequence analysis results

Species	Taxonomy ID	*AMH* (217 bp)	*β-actin* (103 bp)
Bos taurus	9913	100% match*	100% match*
Bos mutus	72004	97.5% match*	100% match*
Bos indicus	9915	100% match*	100% match*
Bison	9900	100% match*	100% match*
Equus caballus	9796	Amplification mismatch	97% match*
Gallus gallus	9031	Amplification mismatch	97% match*
Sus scrofa	9823	Amplification mismatch	100% match*
Mus musculus	10090	Amplification mismatch	90% match*
Rattus norvegicus	10116	Amplification mismatch	90% match*
Oryza sativa	4530	Amplification mismatch	Amplification mismatch
Zea Mays	4577	Amplification mismatch	Amplification mismatch

Table 2. Sample and control Ct values

Sample code	Concentration	Purity		Ct Value	
		260/280	260/230	*AMH*	*β-actin*
Serum 1	3.01	1.24	0.04	nd	35.6
Serum 2	-0.31	-0.01	-0.01	nd	nd
Serum 3	-0.38	-3.36	-0.02	nd	36.57
Serum 4	-0.92	-3.49	3.39	30.50	36.34
Tissue Sample 1	2.10	0.69	0.01	29.85	31.39
gDNA	7.63	0.69	2.88	20.69	28.94

Specificity and Linearity Testing of EvaGreen RT-qPCR-based Reactions

The uMelt simulation indicated that both primer sets generated a single, distinct melting peak, with predicted melting temperatures (Tms) of 93.5 °C for *AMH* and 85 °C for *β-actin*, suggesting high specificity and efficient primer design. Gradient PCR assays confirmed optimal Tas of 70 °C for *AMH* and 65 °C for *β-actin*, with consistent single melting peaks. In subsequent RT-qPCR experiments, melt peaks were obtained at 90 °C for *AMH* and at 82 °C for *β-actin*. Although minor discrepancies were observed between the predicted and experimental Tm, these variations did not substantially affect the interpretation of the results. As illustrated in Figure 2, a comparison of the melting curves of the simulated (left) and experimental (right) results for *AMH* revealed sharp single peaks for both. However, a slight shoulder was observed in the experimental curve, potentially indicating a low-abundance, nonspecific product. For *β-actin*, both simulated and experimental results exhibited uniform, single melting peaks at approximately

84–85 °C. The strong concordance between the computational predictions and laboratory outcomes confirmed that the designed primers were specific and efficient for target DNA detection, with minimal risk of non-specific amplification.

```
661 gaagtgacgt gggagccaac accettgctg aggttccagg agcctccgcc tggaggagcc  ← Forward
721 agcccccag agctggcgct gctggtggtg tacccagggc ctggcctgga ggtcactgtc
781 accggggctg ggctacctgg cacccagagc ctctgcctga ccgcggactc ggacttcctg
841 gccttggtcg tggaccaccc ggaggggcc tggcgccggc ctgggttagc ccttaccctg  ← Reverse
901 cggcgccgtg gaaatggtgc gctcctgagc actgcccagc tgcaggcgct gctgttcggt

  1 ccacgcgtcc gcgccggtcg acaccgcaac cagttcgcca tggatgatga tattgctgcg
 61 ctcgtggtcg acaacggctc cggcatgtgc aaggccggct tcgcgggcga cgatgctccc
121 cgggccgtct tcccgtccat cgtggggcgc ccccggcacc agggcgtaat ggtgggcatg
181 ggccagaagg actcgtacgt gggggatgag gctcagagca agagaggcat cctgaccctc  ← Forward
241 aagtacccca ttgagcacgg catcgtcacc aactgggacg acatggagaa gatctggcac
301 cacaccttct acaacgagct ccgtgtggcc cctgaggagc accccgtgct gctgaccgag  ← Reverse
```

Figure 1. Primer alignment in the reference GenBank sequences of *AMH* (accession no. M13151.1; Cate *et al*., 1986) and *β-actin* (accession no. AY141970.1; Suchyta *et al*., 2004).

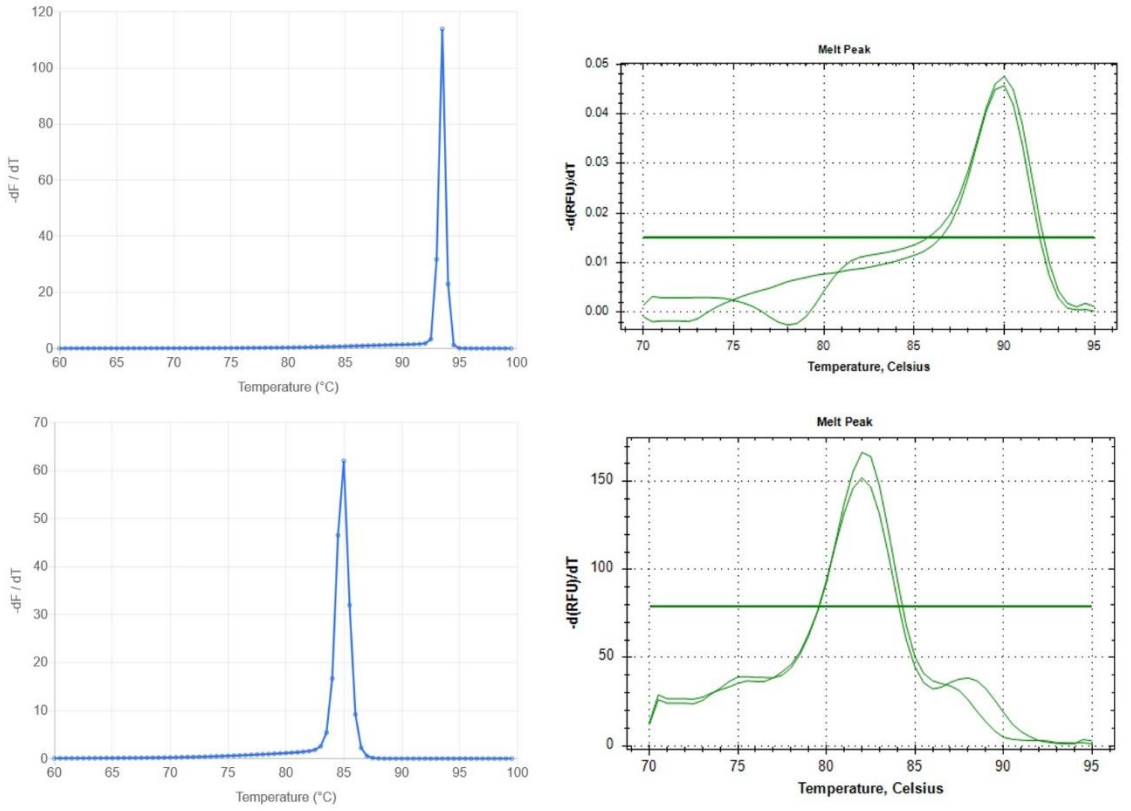

Figure 2. Comparison of the in silico predicted and experimentally derived melting temperatures of *AMH* and *β-actin*.

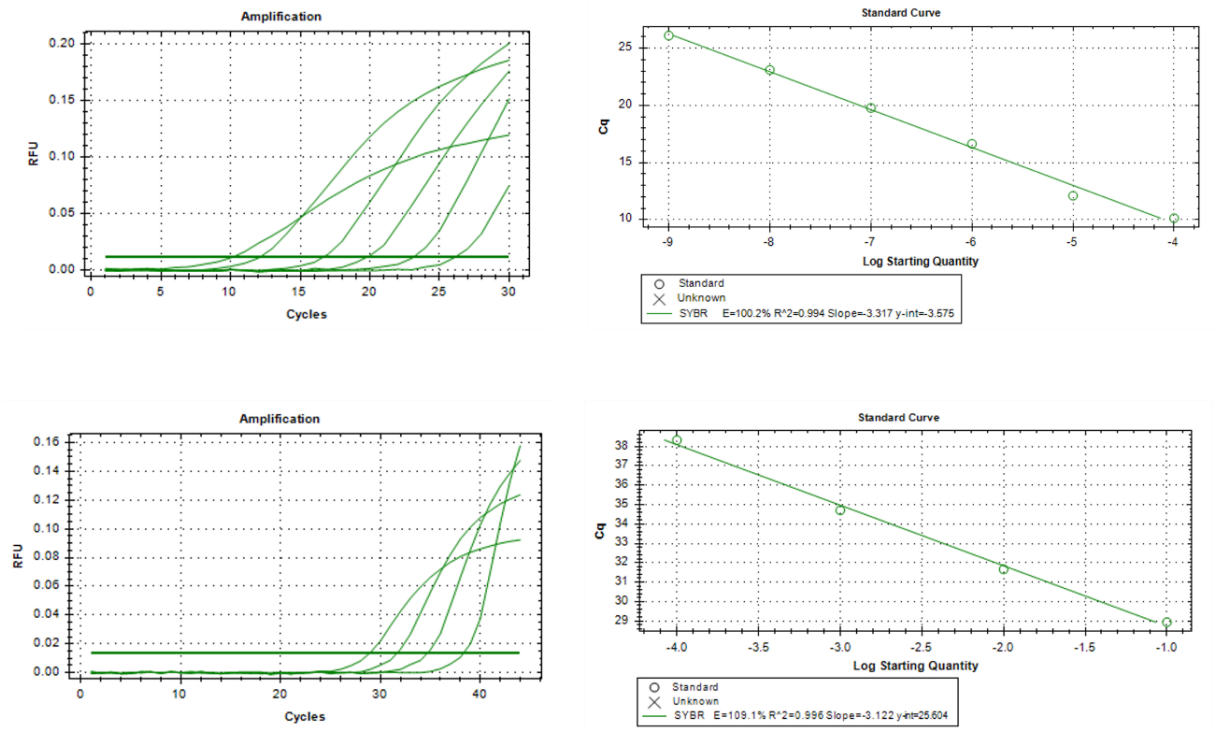

Figure 3. Linearity assessment plots for *AMH* and *β-actin* amplification using RT-qPCR, showing amplification curves (left) and standard curves (right).

Linearity analysis was performed for both the *AMH* and *β-actin* genes using serial dilutions of DNA templates to assess the efficiency and reproducibility of the RT-qPCR assays. For the *AMH* gene, six 10-fold serial dilutions (10^{-1} to 10^{-6}) of DNA fragments were used. Logarithmic regression of the corresponding Ct values yielded a coefficient of determination (R^2) of 0.994, an amplification efficiency (E) of 100.2%, and a regression equation of Y = –3.228x – 1.682. Similarly, the *β-actin* gene was analyzed using genomic DNA serially diluted from an initial concentration of 7.63 ng/µL (dilution range: 10^{-1} to 10^{-4}). The lowest Ct value observed at the 10^{-4} dilution was 38.33. The regression equation was Y = –3.122x + 25.605, with an R^2 value of 0.996 and an E of 109.1%. Figure 3 presents the linearity assessments for *AMH* (top panel) and *β-actin* (bottom panel), where the amplification plots (left panels) show the expected exponential increase in fluorescence, and the standard curves (right panels) display strong linear correlations between Ct values and the logarithmic concentration of the initial DNA templates.

Validation Test using Field Samples

Validation of the RT-qPCR assays was conducted using field-derived biological samples, including both serum and ovarian tissue. Total RNA extraction from these samples revealed low yields and suboptimal purity, as determined by NanoDrop™ spectrophotometric analysis. RNA concentrations were assessed based on absorbance at 260 nm; however, among the three tested serum samples, only one produced a detectable RNA concentration, while the remaining samples yielded either undetectable or negligible values. In general, serum-derived RNA exhibited low quantity and poor purity, as evidenced by A260/280 and A260/230 ratios below the acceptable range (1.8–2.0), indicating contamination with proteins and organic compounds, most likely residuals from the extraction buffer. A strong absorbance peak at 230 nm supported this conclusion. Despite these limitations, RT-qPCR amplification of the *AMH* gene was successful in one out of four serum samples. The *β-actin* gene, used as an internal control to evaluate RNA extraction efficiency, was detectable in most serum samples, albeit with high Ct values, suggesting low template

abundance. One serum sample failed to amplify either gene, indicating severely compromised RNA integrity or an unsuccessful extraction process. In contrast, RNA extracted from tissue samples displayed high integrity and supported efficient amplification, with average Ct values of 29.00 for *AMH* (Table 2).

As a positive control, purified genomic DNA yielded robust amplification, with Ct values of 20.69 for *AMH* and 28.94 for *β-actin*, confirming the accuracy and efficiency of the RT-qPCR protocol (Table 2). These findings underscore the necessity of optimizing RNA purification procedures from serum to minimize contamination and improve amplification success. RNA quality and integrity were further confirmed by 1% agarose gel electrophoresis. All RT-qPCRs reactions were conducted in technical triplicate to ensure reproducibility, and relative gene expression levels were calculated using the 2^−ΔCt method with *β-actin* as the reference gene. No-template controls (NTC) were included in each RT-qPCR run to detect potential contamination or primer-dimer formation. Only reactions that exhibited single, well-defined melting peaks and no amplification in the NTCs were considered valid.

We used a DNA-binding dye-based RT-qPCR method to analyze the expression of *AMH* mRNA in bovine serum. Testing for *AMH*, as a potential molecular biomarker to assess female fertility, offers a potentially more sensitive and non-invasive alternative to conventional approaches. *In silico* analysis was conducted using the uMelt software to assess primer specificity and predict melting behavior before amplification. The designed primers for the *AMH* and *β-actin* genes produced single melting curve profiles, with predicted Tms of 93.5 °C and 85 °C, respectively, indicating high specificity and reaction efficiency. Experimental RT-qPCR validation confirmed similar melting patterns, with slight peak shifts to 90 °C for *AMH* and 82 °C for *β-actin*. The observed minor variations were acceptable, owing to the inherent complexity of in vitro conditions. Nevertheless, the experimental Tm values were closely aligned with the predictions generated using the uMelt software, demonstrating the effectiveness of computational tools in guiding assay optimization before laboratory implementation. Building upon these validation results, the selection of RT-qPCR reagents, particularly the choice of intercalating dye, also plays a crucial role in assay sensitivity and specificity.

While the melting curve analysis revealed sharp, single peaks for both *AMH* and *β-actin*, a subtle shoulder was noted in the *AMH* experimental curve. This may suggest the presence of a low-abundance, non-specific amplicon or primer-dimer formation. However, no amplification was observed in the no-template controls (NTCs), and the melting temperatures remained within the expected range. To further confirm amplification specificity and rule out non-specific products or primer-dimers, we conducted 2% agarose gel electrophoresis of representative RT-qPCR products. The gel images revealed distinct bands at the expected sizes (217 bp for *AMH* and 103 bp for *β-actin*), with no additional bands or smears, thereby validating the specificity of amplification. Considering EvaGreen is a non-specific intercalating dye, such validation is essential to ensure reliable data interpretation. The combination of melting curve analysis and gel electrophoresis confirms that the observed amplification products originated from specific target regions and not from primer artifacts or off-target amplification.

Referring to the latest literature on the role of *AMH* in cattle reproduction, the results of this study further reinforce the relevance of this biomarker in genetic selection and fertility evaluation. EvaGreen was selected as the intercalating dye because of its high binding capacity, thermal stability, and strong fluorescence signal, which contributed to sharp melting curves and high target specificity. These features have previously been reported to enhance gene expression analysis using RT-qPCR (Zhang *et al*., 2015; Taylor *et al*., 2017). Although alternative chemistries, such as TaqMan probes (Daneshian *et al*., 2015) and SYBR Green (Eshel *et al*., 2014; Hu *et al*., 2015), have also been used for *AMH* quantification, the present study

demonstrates that the combination of EvaGreen dye with newly designed primers for *AMH* and *β-actin* provides high amplification efficiency and specificity, making it suitable for serum-based gene expression analysis in cattle. Apart from dye selection, the intrinsic sequence characteristics of target genes, such as GC content, significantly influence RT-qPCR performance.

The *AMH* gene is characterized by a high guanine–cytosine (GC) content (>60%), which strongly influences primer annealing and DNA melting properties. The primers designed for the *AMH* target region had a GC content of approximately 60%, and the amplified region possessed a GC content of 69%. This high GC content contributed to elevated Ta and Tm, as confirmed by both *in silico* and experimental analyses. Due to the strong triple hydrogen bonding between the guanine and cytosine bases, increased thermal stability necessitated higher thermal energy for DNA strand separation. In contrast, the *β-actin* gene, with a GC content of approximately 55% and primer GC content of 50%, exhibited lower Tm and Ta values. These findings underscore the influence of GC content on RT-qPCR assay performance and reinforce the utility of *in silico* tools, such as uMelt, for predicting melting behavior, optimizing primer design, and reducing the need for extensive empirical adjustments, thereby streamlining assay development in terms of cost and time efficiency.

Linearity testing is a critical step in assessing the amplification efficiency and overall performance of a RT-qPCR assay, directly reflecting the accuracy and reliability of nucleic acid quantification. According to the minimum information for the publication of quantitative real-time PCR experiment (MIQE) guidelines, efficiency assessment is essential for validating RT-qPCR protocols (Bustin and Nolan, 2020). An ideal amplification efficiency ranges between 90% and 110%, corresponding to an approximate doubling of the target DNA in each cycle (2^n) (Taylor *et al*., 2010). In our study, the *AMH* assay demonstrated excellent performance, with a reaction efficiency of 100.2%, regression slope of 3.228, and high linearity ($R^2 = 0.994$). Likewise, the *β-actin* assay exhibited a reaction efficiency of 109.1%, a slope of 3.122, and an R^2 of 0.996, indicating highly efficient and reproducible amplification. These findings confirmed that both primer pairs performed optimally and were characterized by high sensitivity, consistent linearity, and accurate quantification. For the *β-actin* target, genomic DNA was utilized as the template. Because of a relatively low initial concentration (7.63 ng/µL), only four 10-fold serial dilutions were prepared, resulting in a maximum Ct value of 38 at the highest dilution. Conversely, to facilitate a broader dynamic range for *AMH*, purified *AMH*-specific DNA fragments were employed as templates, allowing for a six-point, 10-fold dilution series, with successful detection observed up to a 10^{-6} dilution (Ct = 27). The *AMH* primer set hence, yielded a highly efficient and reproducible amplification. The linear amplification behavior and high efficiency of the *AMH* and *β-actin* primers thus support their application in accurate, sensitive, and reproducible molecular detection assays in bovine samples.

We assessed the quality of the extracted RNA using spectrophotometric analysis and RT-qPCR-based validation. Spectrophotometric measurements revealed suboptimal RNA yield and purity across samples. The results indicated organic compound and protein contamination, particularly in the serum samples. Such impurities negatively affect downstream applications, particularly enzymatic reactions, such as RT-qPCR, by inhibiting polymerase activity or compromising nucleic acid stability. These findings highlight the critical need for optimizing RNA purification protocols to ensure the effective removal of inhibitory substances. High-quality RNA maintains the sensitivity, accuracy, and reproducibility of RT-qPCR-based gene expression analysis.

The validation of the test using field-derived samples also confirmed the crucial role of RNA quality in determining RT-qPCR performance. RNA extracted from tissue samples consistently yielded superior amplification results, whereas serum-derived RNA samples produced high Ct values or failed to be amplified. These inconsistencies can be attributed to the low RNA

concentrations and contamination with organic substances, which are likely residues from the extraction reagents. The suboptimal spectrophotometric purity ratios support these inferences. These findings underscore the necessity for rigorous RNA purification and quality control, particularly when working with challenging sample types, such as serum, to ensure reliable RT-qPCR-based detection and quantification.

A marked difference in *AMH* gene expression was observed between tissue and serum samples. The RNA extracted from tissue displayed higher yield, better purity (as reflected in A260/280 ratios), and consistently lower Ct values, enabling reliable amplification of both *AMH* and *β-actin* genes. In contrast, serum-derived RNA was characterized by extremely low concentration and suboptimal purity ratios, often below the acceptable threshold, indicating the presence of protein and organic contaminants.

Serum, as a complex biological fluid, contains lower quantities of intact mRNA due to the absence of cellular components and the presence of endogenous RNases. These RNases can rapidly degrade RNA, especially under suboptimal storage or extraction conditions, resulting in fragmented transcripts that compromise downstream applications. Moreover, the lack of ribosomal RNA in serum hinders the reliable assessment of RNA integrity using conventional electrophoresis methods.

The structural composition of the serum matrix, high protein content, and inhibitory substances such as hemoglobin or lipids further exacerbate RNA instability and affect reverse transcription efficiency. These factors contribute to the reduced detectability of *AMH* mRNA in serum compared to tissue, where cellular integrity is preserved and RNA content is more abundant. The observed discrepancy highlights the need for more refined RNA extraction protocols and possibly pre-amplification steps when working with low-input matrices like serum.

To support quantitative analysis of *AMH* expression, stable reference genes, such as *β-actin*, are required. The *β-actin* gene is widely used as a reference gene for normalizing gene expression data owing to its stable and constitutive expression across various cell types and conditions (Vennapusa *et al*., 2020). In the present study, *β-actin* served as an internal control to assess the quality of RNA extraction. This approach follows a strategy analogous to that employed in SARS-CoV-2 diagnostic protocols, in which nuclear DNA (nDNA) is used to verify the extraction efficiency (Guan *et al*., 2021; Yu *et al*., 2021). The robust performance of the *β-actin* primers and the consistent amplification of the gene across samples support its use as an internal control in RT-qPCR-based gene expression analysis. However, in serum samples, *β-actin* amplification resulted in high Ct values (>35), indicating the possible presence of PCR inhibitors that could interfere with amplification efficiency and potentially lead to false-negative results for *AMH* detection.

Following technical validation of the method, the physiological relevance of *AMH* in the bovine reproductive system warrants further analysis. Physiologically, *AMH* has been extensively studied as a promising biomarker in the bovine reproductive system, particularly in genetic selection, ovarian reserve assessment, and response to environmental stress. *AMH* exhibits a relatively high heritability (h^2 = 0.36–0.46) and stable expression over time, making it a potential indicator in breeding management (Gobikrushanth *et al*., 2017; Mossa and Ireland, 2019). *AMH* concentrations in serum are known to correlate positively with antral follicle count and fertility levels (Jimenez-Krassel *et al*., 2015; Crowe *et al*., 2018), although reference values vary between species approximately 770 pg/mL for *Bos indicus* and 330 pg/mL for *Bos taurus* (Torres-Simental *et al*., 2021).

Although *AMH* levels are relatively stable throughout the estrous cycle and across age groups, environmental factors such as high temperature and humidity can significantly reduce *AMH* levels, particularly under heat-stress conditions. This condition not only reduces *AMH* levels but also disrupts follicle function, gonadotropin hormone synthesis, and decreases oocyte and embryo quality (Gendelman *et al*., 2010; Bernabucci *et al*., 2015). A drastic decrease

in *AMH* levels during the summer has been reported, with a significant reduction from 1018.8 pg/mL to 321.4 pg/mL, correlated with an increase in the temperature-humidity index (Torres-Simental *et al*., 2021). Similarly, Holstein cows experiencing severe heat stress exhibited a decrease in *AMH* levels, from 417.26 ± 4.51 pg/mL to 136.94 ± 4.03 pg/mL, which was closely associated with a decline in pregnancy rates (Gobikrushanth *et al*., 2019).

In addition to environmental influences, physiological differences such as parity status and breed also affect *AMH* concentrations. Primiparous cows tend to have lower *AMH* levels than multiparous cows, which is thought to be related to ovarian maturity and follicular capacity (Gobikrushanth *et al*., 2018). Meanwhile, Holstein cows exhibit lower *AMH* levels compared to beef cows and Jersey cows, despite generally having higher milk production (Ribeiro *et al*., 2014; Hirayama *et al*., 2017). Overall, the combination of genetic stability in *AMH* expression and its sensitivity to environmental factors makes this hormone a relevant candidate biomarker for integration into genomic-based selection programs. Current technological advancements also enable further exploration of functional genes involved in *AMH* expression and adaptation to thermal stress, paving the way for the development of tropical cattle with improved reproductive efficiency (Martínez *et al*., 2021).

The development of *AMH* levels in cattle shows a dynamic pattern during the growth period. Its concentration tends to increase during the first two months of life, decreases at five months of age, and stabilizes again before puberty (Mossa *et al*., 2017). *AMH* levels vary widely among individuals and across age groups, ranging from 6–440 pg/mL in young Holstein cows to over 3,000 pg/mL in adult cows of various breeds during the breeding season (Ribeiro *et al*., 2014; Jimenez-Krassel *et al*., 2015; Alward *et al*., 2021). The consistent relationship between *AMH* levels and *AFC* has established both parameters as reliable predictors of ovarian response to superovulation treatment. Various studies have reported a positive correlation between *AMH* concentration, oocyte-cumulus complex (COC) count, and the number of embryos produced, both in superovulation programs and embryo transfer (Batista *et al*., 2014; Souza *et al*., 2015; Ghanem *et al*., 2016).

The effectiveness of *AMH* and *AFC* as reproductive indicators is also influenced by other factors such as race, age, hormonal protocols, nutritional status, and ovarian reserve (Aziz *et al*., 2017; Besenfelder *et al*., 2020; Jelani *et al*., 2022). Individuals with high *AMH* levels are known to produce a greater number of embryos, even up to twice as many compared to those with low *AMH* levels (Guerreiro *et al*., 2014; Nabenishi *et al*., 2017). Additionally, seasonal variations influence the interpretation of this biomarker; *AMH* levels are reported to be higher in summer than in winter in some regions, so the timing of sample collection must be carefully considered (Xu *et al*., 2021). Overall, the *AMH* hormone offers significant potential as a multifunctional indicator in the reproductive system of cattle, from physiological, genetic, and breeding management perspectives, particularly in addressing environmental stress in tropical regions (Purnama *et al*., 2019).

Unlike conventional approaches, such as measuring *AMH* protein or performing ovarian ultrasound, this study emphasizes the use of a non-invasive molecular approach through the analysis of *AMH* gene expression using RT–qPCR. The findings indicate that the RT-qPCR system based on a combination of *AMH* and *β-actin* primers with EvaGreen dye can produce specific and efficient amplification. However, the quality of RNA from field serum samples remains a challenge. It is important to note that the present study used a very limited number of serum samples (n = 4), which represents a significant limitation in terms of reproducibility and statistical power. This small sample size restricts the ability to draw definitive conclusions regarding population-level variability in *AMH* mRNA expression and may limit the generalizability of the findings. Future research should involve larger and more diverse sample cohorts to validate these preliminary observations and ensure robust statistical analysis. With further optimization of the RNA extraction stage and

expansion of the sample size, this approach may serve as a standard method for assessing ovarian reserve in tropical cattle. Additionally, integrating this approach could complement existing methods in donor selection, reproductive disorder diagnosis, and precision-based local cattle breeding programs. Therefore, this study provides an initial foundation for utilizing *AMH* mRNA expression as a precision molecular tool in developing reproductive strategies and genetic improvement of cattle in tropical regions, as well as strengthening the scientific basis for integrating the RT-qPCR approach into precision livestock fertility monitoring, particularly in tropical environments.

CONCLUSION

This study demonstrated that *AMH* mRNA expression can be detected in serum and tissue of local Indonesian cattle using the EvaGreen dye-based RT-qPCR method, with high primer specificity and amplification efficiency. Despite challenges in serum RNA quality, *β-actin* served as a consistent reference. These results highlight the potential of *AMH* as a non-invasive biomarker for ovarian reserve and reproductive capacity in cattle. Future studies should validate this method with larger cohorts, explore alternative reference genes, evaluate other breeds and environmental conditions to support its integration into tropical livestock breeding programs.

ACKNOWLEDGEMENTS

The authors would like to thank the Institute of Tropical Diseases Universitas Airlangga (ITD), the Board for Standardization of Large Ruminant Agricultural Instruments (BSIP), and the Faculty of Veterinary Medicine Universitas Airlangga for their support and research facilities.

AUTHORS' CONTRIBUTIONS

DMD, HP, SW, and M'AK: Conceptualization, Methodology, and Supervision. DMD, PS, and EML: Methodology, Supervision, Formal analysis, Investigation, Resources, Data curation. SPM and ES: Validation, Supervision, and Formal analysis. TDL and TWS: Resources. M'AK and HP: Formal analysis and Writing - Original Draft/Review and Editing. All authors read, reviewed, and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

REFERENCES

Alward, K. J., & Bohlen, J. F. (2020). Overview of anti-Müllerian hormone (*AMH*) and association with fertility in female cattle. *Reproduction in Domestic Animals*, 55(1), 3–10.

Aziz, R. A., Khalil, A. A. Y., Abdel-Wahab, A., Hassan, N. Y., Abdel-Hamied, E., & Kasimanickam, R. K. (2017). Relationship among circulating anti-Müllerian hormone, insulin like growth factor 1, cadmium and superovulatory response in dairy cows. *Theriogenology*, 100, 72–79.

Barra, G. B., Santa Rita, T. H., Jardim, D. P., Mesquita, P. G., Nobre, C. S., Jácomo, R. H., & Abdalla Nery, L. F. (2019). Genotyping of single nucleotide polymorphisms using allele-specific qPCR producing amplicons of small sizes directly from crude serum isolated from capillary blood by a hand-powered paper centrifuge. *Diagnostics*, 9(1), 88.

Batista, E. O., Macedo, G. G., Sala, R. V., Ortolan, M. D., Sá Filho, M. F., Del Valle, T. A., Jesus, E. F., Lopes, R. N., Renno, F. P., & Baruselli, P. S. (2014). Plasma antimullerian hormone as a predictor of ovarian antral follicular population in *Bos indicus* (Nelore) and *Bos taurus* (Holstein) heifers. *Reproduction in Domestic Animals*, 49(3), 448–452.

Bernabucci, U., Basiricò, L., Morera, P., Dipasquale, D., Vitali, A., Cappelli, F. P., & Calamari, L. U. I. G. I. (2015). Effect of summer season on milk protein fractions in

Holstein cows. *Journal of Dairy Science*, 98(3), 1815–1827.

Berry, D. P., Wall, E., & Pryce, J. E. (2014). Genetics and genomics of reproductive performance in dairy and beef cattle. *Animals*, 8(s1), 105–121.

Besenfelder, U., Brem, G., & Havlicek, V. (2020). Environmental impact on early embryonic development in the bovine species. *Animals*, 14(S1), s103–s112.

Boulton, A. C., Rushton, J., & Wathes, D. C. (2017). An empirical analysis of the cost of rearing dairy heifers from birth to first calving and the time taken to repay these costs. *Animals*, 11(8), 1372–1380.

Bustin, S. A., & Nolan, T. (2020). RT-QPCR testing of SARS-COV-2: A primer. *International Journal of Molecular Sciences*, 21(8), 22.

Cate, R. L., Mattaliano, R. J., Hession, C., Tizard, R., Farber, N. M., Cheung, A., Ninfa, E. G., Frey, A. Z., Gash, D. J., Chow, E. P., Fisher, R. A., Bertonis, J. M., Torres, G., Wallner, B. P., Ramachandran, K. L., Ragin, R. C., Manganaro, T. F., MacLaughlin, D. T., & Donahoe, P. K. (1986). Isolation of the bovine and human genes for müllerian inhibiting substance and expression of the human gene in animal cells. *Cell*, 45(5), 685–698.

Chhalliyil, P., Ilves, H., Kazakov, S. A., Howard, S. J., Johnston, B. H., & Fagan, J. (2020). A real-time quantitative PCR method specific for detection and quantification of the first commercialized genome-edited plant. *Foods*, 9(9), 36.

Costella, A., De Leo, R., Guarino, D., D'Indinosante, M., Concolino, P., Mazzuccato, G., Urbani, A., Scambia, G., Capoluongo, E., Fagotti, A., & Minucci, A. (2018). High-resolution melting analysis coupled with next-generation sequencing as a simple tool for the identification of a novel somatic BRCA2 variant: A case report. *Human Genome Variation*, 5(1), 22.

Crowe, M. A., Hostens, M., & Opsomer, G. (2018). Reproductive management in dairy cows-the future. *Irish Veterinary Journal*, 71, 1–13.

Daneshian, Z., Tehrani, F. R., Zarkesh, M., Zadeh, M. N., Mahdian, R., & Vakili, A. Z. (2015). Antimullerian hormone and its receptor gene expression in prenatally and rogenized female rats. *International Journal of Endocrinology and Metabolism*, 13(1), 56.

Eshel, O., Shirak, A., Dor, L., Band, M., Zak, T., Markovich-Gordon, M., Chalifa-Caspi, V., Feldmesser, E., Weller, J. I., Seroussi, E., Hulata, G., & Ron, M. (2014). Identification of male-specific *AMH* duplication, sexually differentially expressed genes and microRNAs at early embryonic development of Nile tilapia (*Oreochromis niloticus*). *BMC Genomics*, 15(1), 55.

Feres, L. F. R., Siqueira, L. G. B., Palhao, M. P., Dos Santos, L. L., Pfeifer, L. F. M., de Carvalho Fernandes, C. A., & Viana, J. H. M. (2024). Selecting oocyte donors based on anti-Müllerian hormone (*AMH*) concentrations: a critical analysis of using cutoff values as exclusion criterion for an in vitro embryo production program in Gir cattle. *Animal Reproduction Science*, 266, 107491.

Gendelman, M., Aroyo, A., Yavin, S., & Roth, Z. (2010). Seasonal effects on gene expression, cleavage timing, and developmental competence of bovine preimplantation embryos. *Reproduction*, 140(1), 73–82.

Ghanem, N., Jin, J. I., Kim, S. S., Choi, B. H., Lee, K. L., Ha, A. N., & Kong, I. K. (2016). The anti-Müllerian hormone profile is linked with the in vitro embryo production capacity and embryo viability after transfer but cannot predict pregnancy outcome. *Reproduction in Domestic Animals*, 51(2), 301–310.

Gobikrushanth, M., Purfield, D. C., Canadas, E. R., Herlihy, M. M., Kenneally, J., Murray, M., & Butler, S. T. (2019). Anti-Müllerian hormone in grazing dairy cows: Identification of factors affecting plasma concentration, relationship with phenotypic fertility, and genome-wide associations. *Journal of Dairy Science*, 102(12), 11622–11635.

Gobikrushanth, M., Dutra, P. A., Bruinjé, T. C., Colazo, M. G., Butler, S. T., & Ambrose, D. J. (2017). Repeatability of antral follicle counts and anti-Müllerian hormone and their associations determined at an unknown stage of follicular growth and an expected day of follicular wave emergence in dairy cows. *Theriogenology*, 92, 90–94.

Gobikrushanth, M., Purfield, D. C., Colazo, M. G., Butler, S. T., Wang, Z., & Ambrose, D. J. (2018). The relationship between serum anti-Müllerian hormone concentrations and fertility, and genome-wide associations for anti-Müllerian hormone in Holstein cows. *Journal of Dairy Science*, 101(8), 7563–7574.

Guan, B., Frank, K. M., Maldonado, J. O., Beach, M., Pelayo, E., Warner, B. M., & Hufnagel, R. B. (2021). Sensitive Extraction-Free SARS-CoV-2 RNA Virus Detection Using a Novel RNA Preparation Method. *SSRN Electronic Journal*, 9(1), 55.

Guerreiro, B. M., Batista, E. O. S., Vieira, L. M., Sá Filho, M. F. D., Rodrigues, C. A., Netto, A. C., & Baruselli, P. S. (2014). Plasma anti-mullerian hormone: an endocrine marker for in vitro embryo production from *Bos taurus* and *Bos indicus* donors. *Domestic Animal Endocrinology*, 49, 96–104.

Hu, Q., Guo, W., Gao, Y., Tang, R., & Li, D. (2015). Molecular cloning and characterization of *AMH* and dax1 genes and their expression during sex inversion in rice-field eel Monopterus albus. *Scientific Reports*, 5(5), 22.

Hirayama, H., Naito, A., Fukuda, S., Fujii, T., Asada, M., & Inaba, Y. (2017). Long-term changes in plasma anti-Müllerian hormone concentration and the relationship with superovulatory response in Japanese Black cattle. *Journal of Reproduction and Development*, 63, 95–100.

Jelani, G., Kalwar, Q., Kaka, A., Channo, A., Ahmed, A., Soomro, M. H., & Jalbani, Y. M. (2022). Historical background and significance of embryo transfer technology in cattle with its relevant applications. *Pakistan Journal of Zoology*, 55(2), 959–973.

Jimenez-Krassel, F., Scheetz, D. M., Neuder, L. M., Ireland, J. L. H., Pursley, J. R., Smith, G. W., & Ireland, J. J. (2015). Concentration of anti-Müllerian hormone in dairy heifers is positively associated with productive herd life. *Journal of Dairy Science*, 98(5), 3036–3045.

Karl, K. R., Ireland, J. L. H., Clark, Z. L., Tempelman, R. J., Latham, K. E., & Ireland, J. J. (2022). Limitations in use of ovarian reserve biomarkers to predict the superovulation response in small ovarian reserve heifers. *Theriogenology*, 182, 53–62.

Kertz, N. C., Banerjee, P., Dyce, P. W., & Diniz, W. J. (2023). Harnessing genomics and transcriptomics approaches to improve female fertility in beef cattle—a review. *Animals*, 13(20), 3284.

Koca, D., Nak, Y., Sendag, S., Nak, D., Turgut, A. O., Avcılar, T., & Wehrend, A. (2024a). Anti-Müllerian hormone: A novel biomarker for detecting bovine freemartinism. *Reproduction in Domestic Animals*, 59(2), e14542.

Koca, D., Aktar, A., Turgut, A. O., Sagirkaya, H., & Alcay, S. (2024b). Elecsys® *AMH* assay: Determination of Anti-Müllerian hormone levels and evaluation of the relationship between superovulation response in Holstein dairy cows. *Veterinary Medicine and Science*, 10(4), e1509.

Koyama, K., Koyama, T., & Sugimoto, M. (2018). Repeatability of antral follicle count according parity in dairy cows. *Journal of Reproduction and Development*, 64, 535–9.

Krause, A. R. T., Dias, F. C. F., Caunce, S. L., Adams, G. P., Mapletoft, R. J., & Singh, J. (2022). Predictors of the ovarian superstimulatory response and oocyte collection in prepubertal heifers. *Domestic Animal Endocrinology*, 81, 106729.

Kurniawan, M. A., Suwanti, L. T., Mufasirin, M., Suprihati, E., Hastutiek, P., Kusnoto, K., & Priscilia Riwu, K. H. (2025). Morphometric and Molecular Identification of *Eimeria bovis* and *Eimeria zuernii* on Beef Cattle in

Lamongan, East Java, Indonesia. *Jurnal Medik Veteriner*, 8(1), 153–166.

La Marca, A., Ferraretti, A. P., Palermo, R., & Ubaldi, F. M. (2016). The use of ovarian reserve markers in IVF clinical practice: a national consensus. *Gynecological Endocrinology*, 32(1), 1–5.

Manurung, J. J., & Sukohar, A. (2021). Hubungan antara CT value pada test RT-PCR terhadap parameter klinis pasien COVID-19. *Medical Profession Journal of Lampung*, 11(1), 119–124.

Marrella, M. A., & Biase, F. H. (2023). A multi-omics analysis identifies molecular features associated with fertility in heifers (*Bos taurus*). *Scientific Reports*, 13(1), 12664.

Martínez, C. A., Roca, J., & Barranco, I. (2021). Molecular Biomarkers in Animal Reproduction. *Frontiers in Veterinary Science*, 8, 802187.

McGrice, H., Kelly, J. M., Kleemann, D. O., Kind, K. L., Hampton, A. J., Hannemann, P., Walker, S. K., & van Wettere, W. H. E. J. (2020). Plasma anti-Müllerian hormone concentration as a predictive endocrine marker for selection of donor lambs to improve success in juvenile in vitro embryo transfer programs. *Reproduction, Fertility and Development*, 32(4), 383–391.

Meier, S., McNaughton, L. R., Handcock, R., Amer, P. R., Beatson, P. R., Bryant, J. R., & Burke, C. R. (2021). Heifers with positive genetic merit for fertility traits reach puberty earlier and have a greater pregnancy rate than heifers with negative genetic merit for fertility traits. *Journal of Dairy Science*, 104(3), 3707–3721.

Mizuno, R., Yamaguchi, R., Matsuura, K., Ishigami, A., Sakumoto, R., Sawai, K., & Hirayama, H. (2024). Expression and localization of anti-Müllerian hormone and its receptors in bovine corpus luteum. *Theriogenology*, 226, 228–235.

Moolhuijsen, L. M. E., & Visser, J. A. (2020). Anti-Müllerian hormone and ovarian reserve: update on assessing ovarian function. *Journal of Clinical Endocrinology Metabolism*, 105, 3361.

Mossa, F., Jimenez-Krassel, F., Scheetz, D., Weber-Nielsen, M., Evans, A. C. O., & Ireland, J. J. (2017). Anti-Müllerian Hormone (*AMH*) and fertility management in agricultural species. *Reproduction*, 154(1), R1–R11.

Nabenishi, H., Kitahara, G., Takagi, S., Yamazaki, A., & Osawa, T. (2017). Relationship between plasma anti-Müllerian hormone concentrations during the rearing period and subsequent embryo productivity in Japanese black cattle. *Domesticated Animal Endocrinology*, 60, 19–24.

Nawaz, M. Y., Jimenez-Krassel, F., Steibel, J. P., Lu, Y., Baktula, A., Vukasinovic, N., & Tempelman, R. J. (2018). Genomic heritability and genome-wide association analysis of anti-Müllerian hormone in Holstein dairy heifers. *Journal of Dairy Science*, 101(9), 8063–8075.

Purnama, M. T. E., Dewi, W. K., Prayoga, S. F., Triana, N. M., Aji, B. S. P., Fikri, F., & Hamid, I. S. (2019). Preslaughter stress in banyuwangi cattle during transport. *Indian Veterinary Journal*, 96(12), 50–52.

Ribeiro, E. S., Bisinotto, R. S., Lima, F. S., Greco, L. F., Morrison, A., Kumar, A., & Santos, J. E. P. (2014). Plasma anti-Müllerian hormone in adult dairy cows and associations with fertility. *Journal of Dairy Science*, 97(11), 6888–6900.

Souza, A. H., Carvalho, P. D., Rozner, A. E., Vieira, L. M., Hackbart, K. S., Bender, R. W., & Wiltbank, M. C. (2015). Relationship between circulating anti-Müllerian hormone (*AMH*) and superovulatory response of high-producing dairy cows. *Journal of Dairy Science*, 98(1), 169–178.

Suchyta, S. P., Sipkovsky, S., Halgren, R. G., Kruska, R., Elftman, M., Weber-Nielsen, M., Vandehaar, M. J., Xiao, L., Tempelman, R. J., & Coussens, P. M. (2004). Bovine mammary gene expression profiling using a cDNA microarray enhanced for mammary-specific transcripts. *Physiological Genomics*, 16(2), 56.

Taylor, S. C., Laperriere, G., & Germain, H. (2017). Droplet Digital PCR versus qPCR

for gene expression analysis with low abundant targets: From variable nonsense to publication quality data. *Scientific Reports*, 7(1), 23.

Taylor, S., Wakem, M., Dijkman, G., Alsarraj, M., & Nguyen, M. (2010). A practical approach to RT-qPCR-Publishing data that conform to the MIQE guidelines. *Methods*, 50(4), 99.

Torres-Simental, J. F., Peña-Calderón, C., Avendaño-Reyes, L., Correa-Calderón, A., Macías-Cruz, U., Rodríguez-Borbón, A., & Luna-Nevárez, P. (2021). Predictive markers for superovulation response and embryo production in beef cattle managed in northwest Mexico are influenced by climate. *Livestock Science*, 250, 104590.

Vennapusa, A. R., Somayanda, I. M., Doherty, C. J., & Jagadish, S. V. K. (2020). A universal method for high-quality RNA extraction from plant tissues rich in starch, proteins and fiber. *Scientific Reports*, 10(1), 78.

Vidal, L., Álvarez, J., Yáñez, U., Caínzos, J., Muíño, R., Becerra, J. J., & Herradón, P. G. (2024). Evaluation of Anogenital Distance and Anti-Müllerian Hormone Plasmatic Concentration as Potential Phenotypes to Predict Reproductive Performance in Holstein Heifers. *Veterinary Sciences*, 11(10), 495.

Xu, F., Wolf, S., Green, O. R., & Xu, J. (2021). Vitamin D in follicular development and oocyte maturation. *Reproduction*, 161(6), R129–R137.

Yildiz, M., Bastan, I., Guler, S., & Cetin, Y. (2024). Effect of serum anti-Müllerian hormone levels on the superovulation response in Holstein heifers. *Veterinary Medicine and Science*, 10(4), e1507.

Yu, C. Y., Chan, K. G., Yean, C. Y., & Ang, G. Y. (2021). Nucleic acid-based diagnostic tests for the detection SARS-CoV-2: An update. *In Diagnostics*, 11(1), 87.

Zhang, C., Fu, J., Wang, Y., Bao, Z., & Zhao, H. (2015). Identification of suitable reference genes for gene expression normalization in the quantitative real-time PCR analysis of sweet osmanthus (*Osmanthus fragrans* Lour.). *PLoS ONE*, 10(8), 90.

***