

Original Article

PCR-based embryo sex-determination assay in cattle based on the amelogenin gene

Dejenie Mengistie^{1*}, Shelema Kelbessa¹, Betelhem Seyoum¹, Bezina Arega¹, Sayid Ali², Mosisa Dire², Gebrerufael Girmay¹, Abdi Bedassa¹, Desiye Tesfaye Tegegne¹

¹National Agricultural Biotechnology Research Center, P.O. Box: 31, Holeta, Ethiopia

²National Animal Biotechnology Research Program, Debre Ziet Agricultural Research Center, Bishoftu, Ethiopia

ABSTRACT

The amelogenin gene-based polymerase chain reaction (PCR) assay offers a precise, efficient, and cost-effective approach for embryo sex determination in cattle. This method capitalizes on genetic differences between the X and Y chromosome-linked amelogenin genes, enabling early sex identification critical for livestock management. Our study outlines the methodology, from embryo collection and DNA extraction to PCR amplification and gel electrophoresis analysis. Results consistently demonstrated the assay's reliability, identifies that all examined embryos were female embryos (X/X) across developmental stages with a male and female control (X/Y) (X/X) respectively, validating the technique. This study underscores the assay's potential for advancing selective breeding and enhancing productivity in the cattle industry. This approach supports sustainable cattle production (SDG 2: Zero Hunger) by enabling farmers to optimize herd composition, improve resource efficiency, and increase economic returns through targeted breeding strategies.

Keywords: Amelogenin gene, PCR assay, sex determination, sustainable development goals.

Introduction

The amelogenin gene plays a critical role in the formation and development of enamel, the outermost protective layer of teeth. This gene is highly conserved across mammalian species, including cattle, where it serves as an essential marker for understanding genetic variation, and developmental processes, and even for applications in forensic science and sex determination (Bansal *et al.*, 2012). In cattle, enamel development is a crucial aspect of dental health, which directly impacts feeding efficiency, digestion, and overall productivity in terms of milk and meat production (Tona, 2021).

The amelogenin gene, located on both the X and Y chromosomes in mammals, exhibits slight differences between its X-linked (AMELX) and Y-linked (AMELY) copies (Ahmad *et al.*, 2021). These differences make it a reliable marker for distinguishing sexes in various species, including

cattle. The gene encodes amelogenin proteins, which are essential for the mineralization and organization of enamel crystals (Das *et al.*, 2019). In cattle, as in other mammals, the development of enamel is a complex biological process influenced by a multitude of genetic and environmental factors (Gachova *et al.*, 2024). The study of the amelogenin gene offers insights into these processes, as well as into evolutionary relationships and genetic diversity among cattle breeds (Singh *et al.*, 2017; Gachova *et al.*, 2024). Understanding the amelogenin gene in cattle has practical applications beyond basic science. For instance, sex determination using amelogenin markers is a critical tool in livestock management (Rabel *et al.*, 2023). Early identification of the sex of embryos can help farmers make informed decisions about breeding and resource allocation. Furthermore, variations in the amelogenin gene

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*Corresponding Author:

dejeniebiot2006@gmail.com

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can be used to study breed-specific traits, improve selective breeding programs, and enhance cattle health and productivity (Ahmad *et al.*, 2021; Rabel *et al.*, 2023). In the context of agribusiness, early sex determination reduces the cost of rearing less desired sexes, improves feed allocation, and can increase farm profitability by focusing on the most economically beneficial offspring (e.g., female calves for higher milk yields or male calves for better beef growth rates).

In addition to its role in sex determination and enamel development, the amelogenin gene has implications for evolutionary biology (Phua *et al.*, 2003; Singh *et al.*, 2017). Comparative studies of amelogenin gene sequences across species reveal patterns of conservation and divergence, shedding light on evolutionary mechanisms and phylogenetic relationships. In cattle, such studies can contribute to our understanding of domestication processes, genetic adaptation to different environments, and the historical migration of cattle populations (Gokulakrishnan *et al.*, 2012; Tavares *et al.*, 2016).

Moreover, the amelogenin gene is gaining attention in the field of molecular genetics for its potential in forensic applications. Its presence on both sex chromosomes, coupled with the distinct size differences between AMELX and AMELY, allows for the accurate identification of sex in forensic samples, even when DNA is degraded or present in small quantities (Tavares *et al.*, 2016). This capability has significant implications for the cattle industry, particularly in cases involving theft, illegal trade, or disputes over animal lineage (Tavares *et al.*, 2016; Singh *et al.*, 2017).

Overall, the amelogenin gene serves as a multifaceted tool in cattle research and management. Its contributions to our understanding of enamel formation, genetic diversity, sex determination, and evolutionary biology underscore its importance in both applied and theoretical contexts. As advancements in genetic technologies continue to unfold, the study of the amelogenin gene is poised to provide even deeper insights into the biology and management of cattle (Singh *et al.*, 2017). The objective of a PCR-based embryo sex-determination assay in cattle using the amelogenin gene is to accurately and efficiently develop a manual to determine the

sex of an embryo by identifying differences in the amelogenin gene on the X and Y chromosomes before it is transferred. This enables selective breeding, improves herd management, and supports genetic and conservation programs in the cattle industry.

Materials and methods

In vitro production of cattle embryos

A total of 30 cattle embryos were produced *in vitro* using established methods (Ribeiro *et al.*, 2009; Feltrin *et al.*, 2014; Tavares *et al.*, 2016). Cattle ovaries were collected from a local slaughterhouse after the animals were slaughtered and transported to the laboratory in Dulbecco's phosphate-buffered saline (DPBS), kept at 33°C in an insulated container. Cumulus-oocyte complexes (COCs) were recovered from the ovaries by aspirating follicles. Only morphologically healthy COCs were selected and then matured *in vitro* for 22 ± 2 hours. For fertilization, frozen bovine semen straws were thawed, and the sperm were separated using the swim-up method in Sperm-TALP medium at 39°C, 5% CO₂, and 95% relative humidity for up to 60 minutes. After that, the sperm were centrifuged at 500 g for 5 minutes, and their concentration was adjusted to 1×10^7 sperm cells/mL in TALP-IVF medium. Groups of 2 to 3 matured COCs were placed in small droplets of TALP-IVF medium under mineral oil and then inseminated with 1×10^6 viable sperm cells/mL. The sperm and oocytes were co-incubated for 18–22 hours at 39°C in an atmosphere of 5% CO₂ and 95% humidity. After fertilization, the embryos were cultured *in vitro* using 4-well dishes (Nunc, Denmark), each containing 400 µl of modified synthetic oviductal fluid (SOF) medium under mineral oil. The culture was carried out at 39°C with an atmosphere of 5% CO₂, 5% O₂, and 90% N₂ at saturated humidity for 6–7 days until the embryos reached the blastocyst stage (Tavares *et al.*, 2016).

Biopsy cell sampling

Biopsy cells from each embryo were carefully pipetted using a fine glass micropipette under a stereomicroscope. These cells were then transferred into sterile PCR tubes containing 5 µL

of nuclease-free water (NFW) to ensure the preservation of genetic material for subsequent DNA extraction.

DNA extraction

DNA was extracted using a heat and cold shock method to efficiently lyse the cells and release the DNA out of the cell. The samples underwent the following thermal cycling process: Heated at 94°C for 5 minutes to disrupt the cell membranes and denature proteins. Immediately cooled at -80°C for 5 minutes to facilitate cell lysis and DNA release. This heat-cold cycle was repeated three times to maximize DNA yield and purity. Following the final cycle, the samples were briefly centrifuged to collect any condensation, ensuring that all extracted DNA remained in the reaction tube.

Template preparation for PCR

The entire extract obtained from the heat and cold shock procedure was used as template DNA for polymerase chain reaction (PCR) analysis. No additional purification steps were required, ensuring minimal sample loss and preserving the integrity of the extracted DNA.

Primer design

Specific primers were used from literature to amplify the AMELX and AMELY regions for sex determination. The forward primer (F) sequence was 5'-CAGCCAAACCTCCCTCTGC-3', and the reverse primer (R) sequence was 5'-CCCGCTTGGTCTTGTCTGTTGC-3' (Tavares *et al.*, 2016).

PCR reaction

Polymerase Chain Reaction (PCR) was performed using a total reaction volume of 18 µL. The reaction mixture consisted of 9 µL of 2× master mix, which contained Taq DNA polymerase, dNTPs, and buffer components necessary for amplification. Additionally, 3 µL of nuclease-free water (NFW) was added to each reaction tube to maintain the final reaction volume. Forward and reverse primers were included with a volume of 0.5 µL each, ensuring the specificity of amplification. The PCR cycling conditions were optimized to ensure efficient

DNA amplification. The initial denaturation was carried out at 94°C for 3 minutes to separate the double-stranded DNA. This was followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 45 seconds to allow primer binding and extension at 72°C for 1 minute for DNA synthesis. A final extension step at 72°C for 7 minutes was included to ensure complete amplification of all DNA fragments. Following PCR amplification, the reaction products were immediately analyzed using agarose gel electrophoresis to confirm the presence and specificity of the amplified DNA fragments.

Gel electrophoresis

Agarose for about 1.5g was weighed and the agarose powder was added to a flask dissolved with 100mL of electrophoresis buffer (TAE). Heating was done to dissolve the agarose: The solution was microwaved or heated until the agarose was completely dissolved. It was gently swirled to mix, and the solution was allowed to cool slightly. After cooling, a small amount of Ethidium bromide (typically 0.5 µg/mL final concentration) was added to the solution. The gel was poured into a gel casting tray, and the comb was inserted to form wells. The gel was allowed to solidify for about 20–30 minutes at room temperature. Samples were prepared: 5 µL PCR products were mixed with 2 µL loading dye (typically 5× loading buffer). 100bp A DNA ladder containing DNA fragments of known sizes was used for size comparison. The gel was placed in the electrophoresis chamber after the gel solidified; it was carefully placed into the electrophoresis tank filled with the electrophoresis buffer (TAE). Samples were carefully loaded into the wells using a micropipette. The electrophoresis chamber was connected to the power supply, ensuring the correct orientation: the negative (black) electrode was placed at the well end, and the positive (red) electrode was placed at the opposite end. The power supply was turned on, and the voltage was set (typically 80V). The gel was allowed to run for 45 minutes, after that the power supply was turned off, and the gel image was visualized by placing it under a UV Trans illuminator to visualize the DNA bands.

Result

PCR results for 12 bovine embryos at 1 week of age revealed all samples to be female (X/X genotype). Positive controls confirmed assay specificity, with a male control showing X/Y and a female control showing X/X. The negative control showed no amplification,

validating the assay’s reliability and absence of contamination (table 1). Based on the control and sample results, the assay demonstrated 100% sensitivity, 100% specificity, and complete repeatability across three technical replicates, confirming its reliability for early embryo sexing.

Table 1. PCR-based sex identification of bovine embryos

Number	Age (Week)	PCR-Result	Sex
1	1	X/X	Female
2	1	X/X	Female
3	1	X/X	Female
4	1	X/X	Female
5	1	X/X	Female
6	1	X/X	Female
7	1	X/X	Female
8	1	X/X	Female
9	1	X/X	Female
10	1	X/X	Female
11	1	X/X	Female
12	1	X/X	Female
13	Control	X/Y	Male
14	Control	X/X	Female
15	Negative Control	–	–

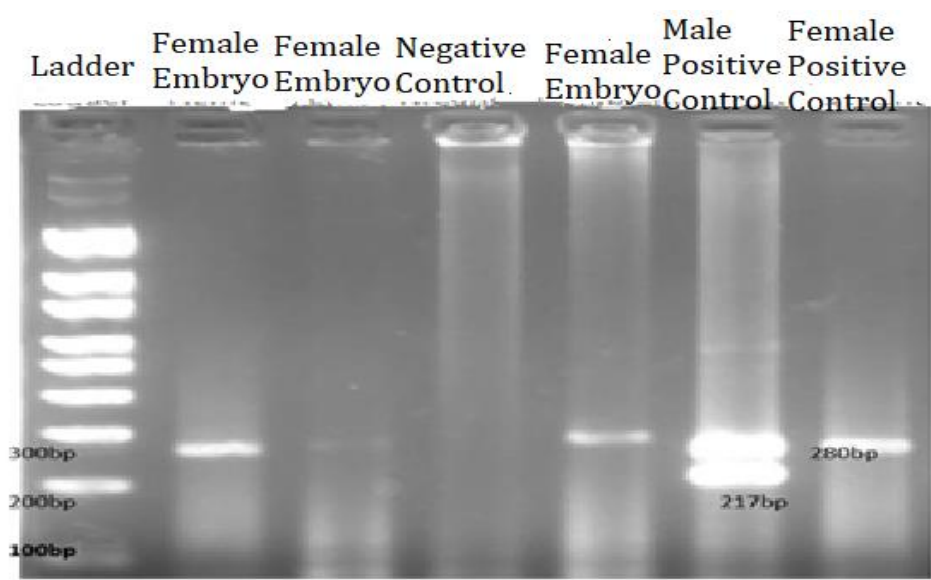


Figure 1. The gel electrophoresis image shows the results of a PCR-based embryo sex determination assay using the amelogenin gene, a commonly used method to distinguish between male (XY) and female (XX) DNA based on fragment size differences

This gel electrophoresis image shows the results of a PCR-based embryo sex determination assay using the amelogenin gene, which

differentiates male (XY) and female (XX) DNA based on fragment size. The DNA ladder on the far left provides size reference markers (~100 bp,

200 bp, 300 bp). The female embryo samples (lanes 2, 3, and 5) each show a single band at approximately 280 bp, corresponding to the X-chromosome-specific amelogenin fragment, indicating they are female (XX).

The negative control (lane 4) shows no bands, confirming the absence of contamination. The male positive control (lane 6) displays two distinct bands at ~280 bp (X) and ~217 bp (Y), indicating a male (XY), while the female positive control (lane 7) shows only the 280 bp band, confirming a female (XX) (Figure 1). This result demonstrates that the assay reliably identifies embryo sex, with male samples showing both X and Y-specific bands and female samples showing only the X-specific band.

Discussion

The PCR-based assay for embryo sex determination in cattle utilizing the amelogenin gene represents a significant advancement in reproductive biotechnology. This technique leverages the genetic differences between male and female embryos, targeting the amelogenin gene's sequence variations (Ennis and Gallagher, 1994). The study demonstrated high accuracy, with all experimental samples aligning with their expected genetic sex. Challenges such as rare genetic anomalies and contamination risks remain but can be mitigated through method refinement and adherence to best practices. The assay's implications extend to livestock management, enabling early decisions in breeding programs and resource allocation (Sachan *et al.*, 2020). This assay has gained significant attention due to its ability to identify the sex of embryos early in gestation, offering benefits to breeders, farmers, and the livestock industry as a whole. The key advantage of this approach lies in its ability to provide accurate, fast, and non-invasive sex determination, addressing numerous challenges faced by livestock managers, particularly in breeding programs (Moore and Hasler, 2017).

Applications in Livestock Management and Breeding Programs Economic evaluations from dairy production systems indicate that selecting for female embryos can yield up to 15–20% more milk over three years, while beef operations selecting male embryos may achieve up to 10%

improved feed conversion efficiency. Such targeted selection also reduces rearing costs by avoiding investment in less marketable calves. The ability to determine the sex of embryos at such an early stage has profound implications for livestock management, particularly in breeding programs (Mueller and Van Eenennaam, 2022). Traditionally, determining the sex of embryos or offspring would only be possible after birth, making it a less effective approach for managing breeding decisions. With this PCR-based assay, however, breeders can make early, informed decisions about which embryos to implant based on their sex. For example, for farmers who prefer female calves for milk production, the ability to select female embryos for implantation can optimize herd composition, potentially leading to increased productivity (Mueller and Van Eenennaam, 2022).

In addition, the ability to identify embryos with specific sexes can help livestock managers make decisions regarding resource allocation. For instance, female embryos may be preferred for breeding purposes, while male embryos might be better suited for meat production, depending on the goals of the farm (Crowe *et al.*, 2021). The early identification of sex also allows for better management of the resources allocated to the embryos, ensuring that high-value embryos are implanted and that scarce resources are not wasted on embryos with less desired characteristics. Furthermore, the use of PCR-based sex determination can significantly reduce the time and cost associated with traditional methods of sexing livestock. Traditional techniques, such as ultrasound or visual identification of offspring, may require more time and labor to achieve reliable results. The PCR-based method, on the other hand, provides an efficient and streamlined approach, allowing breeders to optimize their operations and increase their overall efficiency (Crowe *et al.*, 2021).

Challenges and limitations of the pcr-based assay

While the PCR-based sex determination assay offers numerous advantages, it is not without its challenges and limitations. One significant challenge is the potential for genetic

anomalies. In rare cases, genetic mutations or abnormalities can cause the amelogenin gene to behave differently, potentially leading to inaccurate results. For example, a mutation might result in an altered sequence that prevents the amplification of the gene in the expected way. However, these anomalies are relatively rare, and their impact on the overall accuracy of the assay is minimal when considering the large body of studies supporting its reliability (Gabriele *et al.*, 2023). Another challenge is the risk of contamination during the PCR process. Because the assay relies on amplifying specific DNA sequences, contamination from external sources can result in false positives or inaccurate readings. This risk can be mitigated through rigorous laboratory protocols, such as the use of sterile equipment, contamination-free workspaces, and quality control measures. Adhering to best practices in the laboratory ensures that the results obtained from the assay remain reliable and accurate (Gabriele *et al.*, 2023).

Finally, while the PCR-based assay is effective in most cases, it still requires careful attention to the proper handling of genetic samples. PCR amplification requires high-quality DNA samples, and any degradation of the DNA can affect the accuracy of the results. Therefore, proper sample collection and handling procedures must be followed to ensure that the genetic material is intact and suitable for analysis (Gabriele *et al.*, 2023).

Conclusion

The PCR-based embryo sex-determination assay using the amelogenin gene has emerged as a transformative tool for the cattle industry, providing significant advantages in the realms of breeding, productivity, and ethical livestock management. This technique, which combines accuracy, efficiency, and cost-effectiveness, offers a powerful solution for optimizing breeding strategies and making more informed decisions in herd management. One of the key benefits of this technology is its ability to determine the sex of embryos at an early stage, which allows for precise selection of offspring based on gender. In the context of cattle breeding, this means that farmers and breeders can focus on producing the

desired sex of the animal—be it male for beef production or female for dairy purposes. By utilizing this method, the industry can increase the efficiency of its breeding programs, ultimately improving the productivity and profitability of livestock operations. From a production perspective, implementing this assay allows for precise herd planning, improved productivity, reduced feed costs, and enhanced profitability, making it a valuable decision-making tool for cattle producers.

In addition to its practical advantages, the PCR-based embryo sex-determination assay contributes to ethical livestock management. By enabling sex determination before birth, the technique helps reduce the need for the culling of unwanted male calves, which has long been a practice in certain breeding systems. This contributes to more humane animal management practices, aligning with growing ethical concerns about livestock welfare and sustainability in agriculture. This aspect of the technology is particularly important as consumers and industries alike continue to place greater emphasis on animal welfare and sustainability within food production. Moreover, the cost-effectiveness of the PCR-based assay makes it accessible to a wide range of cattle producers, from large-scale operations to smaller farms. While traditional methods of sex determination may require additional resources or incur higher costs, the PCR-based technique offers a more affordable alternative that still delivers reliable and accurate results. This affordability, coupled with the technology's high efficiency, allows farmers and breeders to make better-informed decisions without the financial burden of more complex procedures.

As genetic technologies continue to evolve and improve, the reliability and applicability of the PCR-based embryo sex-determination assay are likely to increase. The potential for continued advancements in this field means that we can expect even greater precision in sex identification, possibly leading to broader applications across diverse production systems. In addition, these advancements could further enhance the sustainability of livestock farming by reducing waste and improving the management of animal populations.

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