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Non-Invasive Prenatal Detection of Y-Chromosome Sequences (SRY, AMEL) in Cattle Using Maternal Plasma Cell-Free Fetal DNA

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Abstract: The detection of cell-free fetal DNA (cfDNA) in maternal plasma provides a non-invasive approach for early prenatal diagnosis and fetal sex determination in cattle. The present pilot study aimed to evaluate the feasibility of this method under routine laboratory conditions using plasma samples from pregnant cows. Eight plasma samples were collected from clinically confirmed pregnant cows during mid-gestation. cfDNA was isolated from 1 mL of plasma using a modified protocol based on the QIAamp DNA Mini Kit. Conventional PCR assays were applied to amplify Y-chromosome-specific SRY and AMEL gene sequences, while a known male bovine DNA sample served as positive control. The amplification of a 162 bp fragment of the SRY gene or a double band pattern for the AMEL gene (241 bp for X and 178 bp for Y) was interpreted as indicative of a male fetus. Among the analyzed samples, one cow showed positive amplification for both SRY and AMEL-Y sequences, confirming the presence of a male fetus, whereas the remaining seven samples were negative for Y-specific amplicons, consistent with female pregnancies. These findings confirm the applicability of circulating cfDNA from maternal plasma for non-invasive detection of bovine fetal sex, even when a limited sample number is used. Although this preliminary experiment demonstrates the feasibility of the method, further optimization and validation using a larger population and quantitative PCR analysis are required to determine diagnostic accuracy and to establish cfDNA-based fetal sexing as a reliable routine procedure in cattle breeding programs.

Keywords: Cell-free fetal DNA; Y-chromosome markers; Fetal sex determination; Cattle; PCR; Non-invasive prenatal testing

• Introduction

The discovery of cell-free fetal DNA (cfDNA) in maternal plasma revolutionized non-invasive prenatal diagnosis by enabling detection of fetal genetic material without invasive sampling procedures (Lo, 2005; Chiu & Lo, 2010). In humans, cfDNA testing has become an established method for fetal sex determination and detection of chromosomal or monogenic disorders (Skrzypek & Hui, 2017). In cattle, early identification of fetal sex holds major economic importance, allowing optimization of breeding strategies, improved herd management, and targeted embryo transfer or insemination programs (Muthusamy et al., 2016).

Unlike invasive techniques or late-term ultrasonography, cfDNA-based analysis provides a safe and repeatable tool that can be applied in early gestation using small volumes of maternal plasma. Fetal cfDNA originates mainly from apoptotic trophoblast cells and circulates as short DNA fragments of approximately 150–200 bp (Repiská et al., 2012).

Although the synepitheliochorial bovine placenta limits the passage of fetal DNA compared to humans (Lemos et al., 2011), several studies have successfully detected Y-chromosome-specific sequences such as SRY and AMEL-Y using PCR amplification (Stanghellini et al., 2006; Tong et al., 2007; Muthusamy et al., 2016).

The present study aimed to evaluate the feasibility of non-invasive bovine fetal sex determination under standard laboratory conditions by detecting SRY and AMEL gene fragments from maternal plasma cfDNA through endpoint and quantitative PCR assays.

• Material and method

Sample collection and DNA extraction

Blood was collected from eight pregnant cows into sterile EDTA tubes. Plasma was separated by centrifugation (1,000 × g for 10 min, followed by 2,000 × g for 5 min) and stored at –80 °C until analysis. Cell-free DNA was extracted from 300 µL of plasma using the NucleoSpin DNA Forensic Kit (Macherey-Nagel, Düren, Germany), following the manufacturer's protocol adapted for liquid samples. DNA purity and concentration were checked spectrophotometrically (NanoDrop 8000, Thermo Scientific).

Endpoint PCR

Two specific markers were used for fetal sex determination: the SRY (Y-chromosome-specific) and AMEL (amelogenin) genes. PCR amplification was performed in 25 µL reactions containing 12.5 µL GoTaq® Green Master Mix (Promega, Madison, WI, USA), 10 µM of each primer, and cfDNA template (> 20 ng). The reactions were run on an Eppendorf Mastercycler pro S thermal cycler.

For both primer sets, the PCR profile consisted of: 94 °C – 5 min; 38 cycles of 94 °C – 30 s, 56 °C – 30 s, 72 °C – 30 s; final extension 72 °C – 5 min.

Amplified products were separated on 2 % agarose gels in TAE buffer, visualized under UV illumination, and compared with a 100 bp molecular weight ladder. A male control (MC) and DNA ladder (M) were included in each run.

Quantitative PCR (qPCR)

For confirmation, the SRY gene was quantified using a PowerUp SYBR Green Master Mix (Applied Biosystems) on an ABI real-time PCR system. Each 20 µL reaction contained 4 µL of cfDNA, 10 µL of SYBR Green mix, and 5 pmol of each primer. Cycling conditions were as recommended by the kit. Fluorescence amplification and melting-curve analyses were used to confirm specificity of the amplicons.

• Results and discussions

Cell-free DNA was successfully extracted from all eight plasma samples. Endpoint PCR using the AMEL gene revealed two distinct bands (~241 bp for AMEL-X and ~178 bp for AMEL-Y) in one cow and in the male control, confirming a male fetus. The remaining seven cows exhibited a single AMEL-X band, indicating female fetuses. Similarly, amplification of the SRY gene (~162 bp) was detected only in Cow 1 and in the male control (Figures 1–2).

Quantitative PCR confirmed these findings: only one sample (Cow 1) showed a sigmoidal amplification curve and a specific melting peak at 82 °C identical to the male control (Figures 3–4), consistent with male fetal DNA. The other samples lacked amplification, correlating with female pregnancies.

These results are in agreement with Muthusamy et al. (2016), who reported 88.9 % accuracy for conventional PCR and 96.3 % for qPCR in bovine fetal sexing using SRY and AMEL markers. The present data confirm that even minimal cfDNA volumes (300 µL plasma) are sufficient for detection under standard laboratory conditions. While sensitivity is inherently lower than in humans due to the reduced fetal DNA fraction in bovine plasma, the observed amplification specificity supports the robustness of the method (Repiská et al., 2012; Everett & Chitty, 2015).

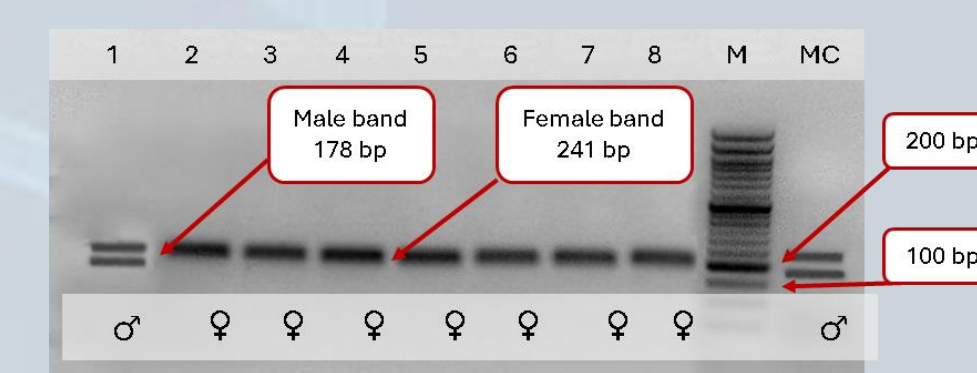


Figure 1. AMEL test on agarose gel

The AMEL (amelogenin) test targets both X- and Y-specific amelogenin gene fragments. The male control (MC) and Cow 1 display two bands—one corresponding to the AMEL-X and one to the AMEL-Y allele—confirming the presence of a male fetus. Samples from Cows 2–8 show only the AMEL-X band, consistent with female fetuses. The DNA ladder (M) enables verification of expected product sizes (~241 bp for AMEL-X and ~178 bp for AMEL-Y).

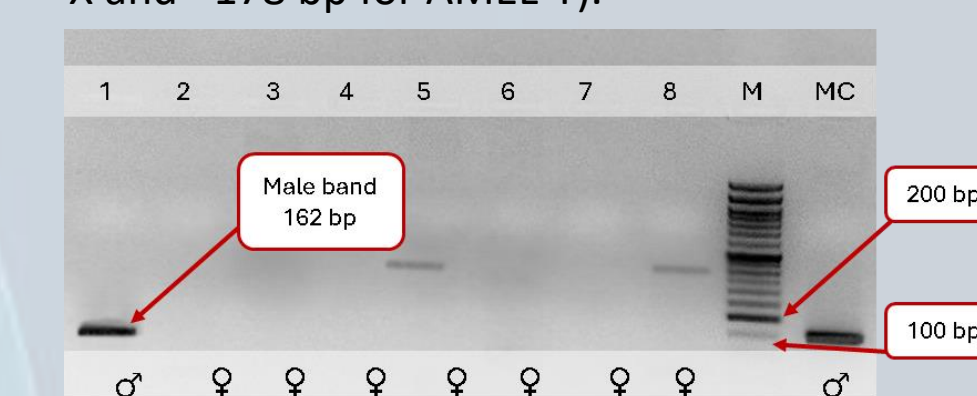


Figure 2. SRY test on agarose gel

Agarose gel electrophoresis shows the SRY-specific band (~160 bp) in Cow 1 and in the male control (MC). Lanes corresponding to Cows 2–8 do not display any visible band at this position, indicating the absence of Y-chromosome DNA and, therefore, a female fetus. The DNA ladder (M) confirms correct fragment size alignment.

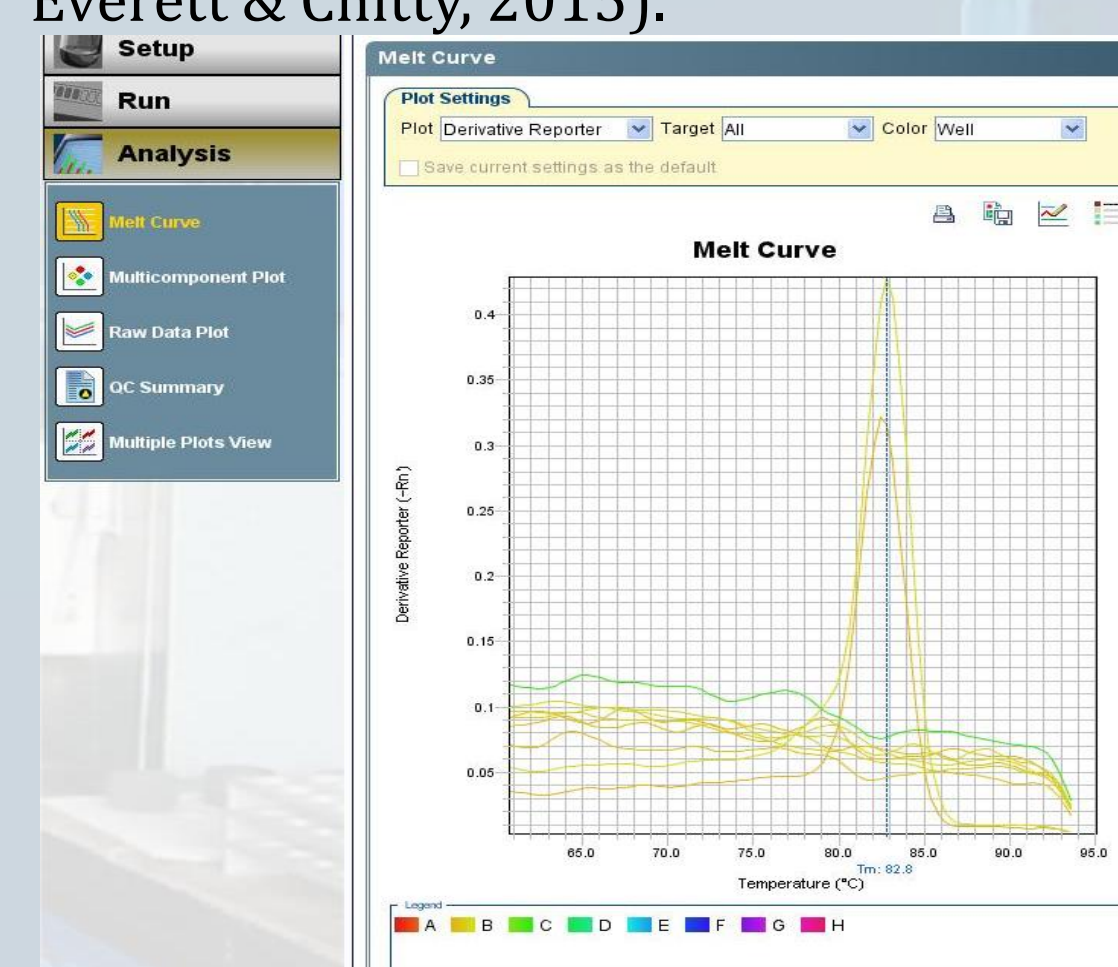


Figure 4. Melting curve of SRY qPCR reaction

The melting curve analysis reveals a single, sharp peak at the expected melting temperature (T_m) for the SRY amplicon (~82 °C). The Cow 1 sample and male control (MC) display identical peaks, confirming specific amplification of the SRY fragment. No additional peaks are observed for the remaining cows, indicating the absence of nonspecific products and supporting the male-specific result only in Cow 1.

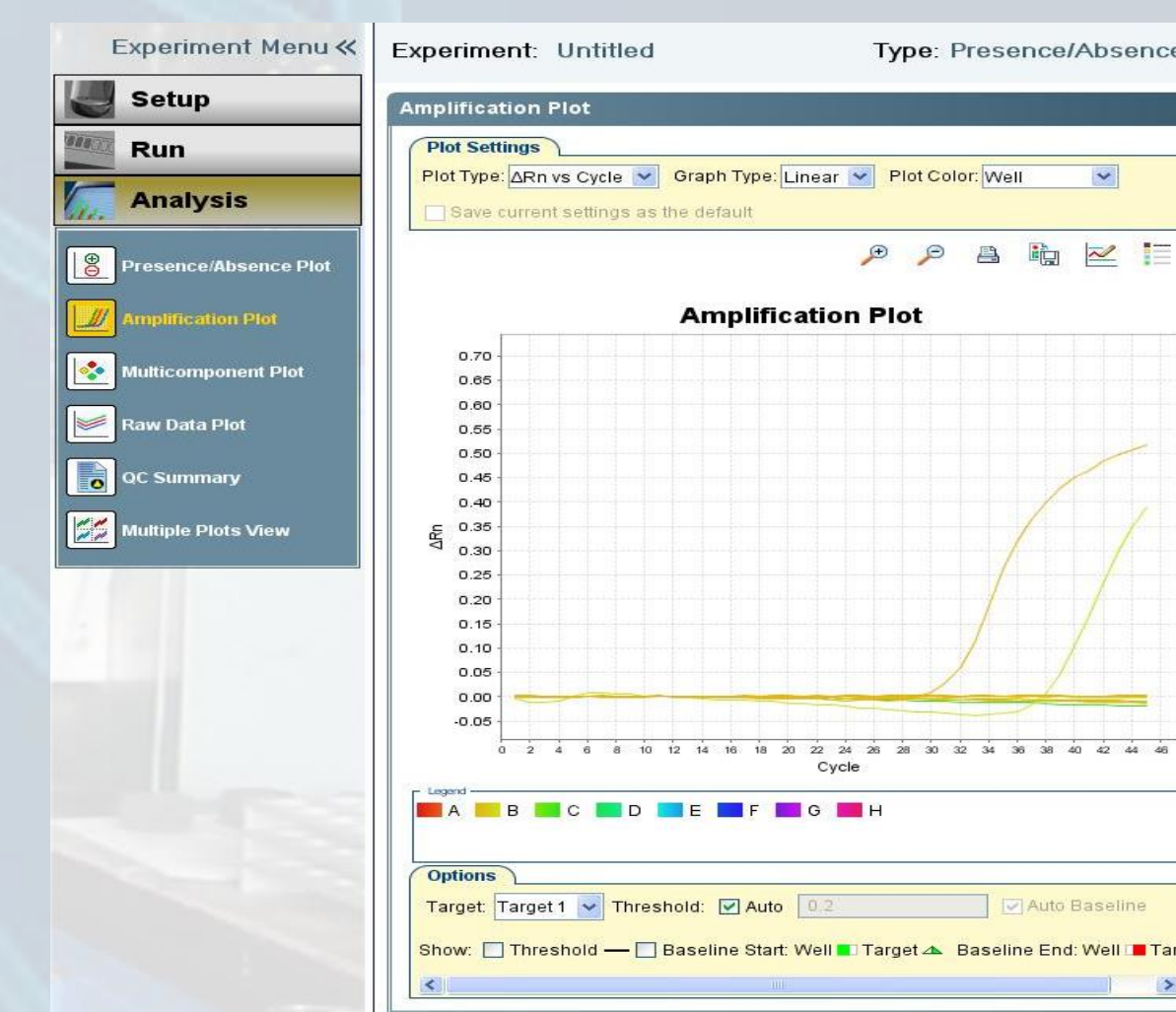


Figure 3. Amplification plot of SRY qPCR reaction

The amplification plot illustrates the detection of the SRY gene in plasma DNA extracted from eight pregnant cows. Only one sample (Cow 1) exhibited a distinct amplification curve overlapping with the male control (MC), confirming the presence of the Y-chromosome-specific SRY sequence. All other samples (Cows 2–8) remained below the fluorescence threshold, consistent with female fetuses. The DNA ladder (M) was not included in this plot, as qPCR signals are shown in relative fluorescence units versus cycle number.

• Conclusions

This preliminary study demonstrates that fetal sex can be accurately determined from maternal plasma cfDNA in cattle using conventional and real-time PCR amplification of SRY and AMEL genes. The method provides a practical, non-invasive alternative to invasive sampling techniques and shows strong concordance with literature-reported diagnostic accuracies. Future work should focus on increasing sample size, optimizing cfDNA yield, and establishing qPCR-based quantification standards to enable its integration into routine bovine reproductive management.