Veterinary Integrative Sciences 2023; 21(3): 657 - 665 DOI; 10.12982/VIS.2023.047

Vet Integr Sci

Veterinary Integrative Sciences

ISSN; 2629-9968 (online)

Website; www.vet.cmu.ac.th/cmvj

Research article

Cattle fetal sex determination using cell-free fetal DNA from maternal blood in the Mekong Delta, Vietnam

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Abstract

Cell-free fetal DNA (cffDNA) from fetal calves was isolated in the bovine maternal plasma in the fifth week of pregnancy, accounting for around 10% of the total. Recently, cffDNA has been used as a non-invasive prenatal screening technique for aneuploidy, genetic disorders in humans, early pregnancy diagnosis, and animal fetal sex determination. This study's objective was to identify the presence of cffDNA in the blood of 13 dairy cows (11 pregnant, 2 non-pregnant, and 1 unmarred) gathered from farms in Can Tho, the Mekong Delta, Vietnam. Plasma / Serum cfc-DNA Purification Midi Kit (Product # 55600) was used to separate cffDNA. Two PCR-designed primers named SRY-137 and GAPDH-109 were employed to amplify the Y-chromosome-specific sequences SRY and the housekeeping gene GAPDH, respectively. The fetal sex was indicated by PCR data corresponding to the calf's sex at birth in 11 cases. The study's findings first proved the existence of cell-free fetal DNA circulating in the plasma of a pregnant cow in Vietnam and established the calf's sex using simple, high-precision procedures. The use of cffDNA for sex screening brings up the possibility of quickly determining the gender and screening for genetic diseases in a big herd of cattle and animals with long gestation periods.

Keywords: : Cell-free fetal DNA, Dairy cow, GAPDH, PCR, Plasma, SRY

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Funding; This research was supported by funding from the Scientific Research Fund of Can Tho University, project code: TSV2019-139.

Article history; *received manuscript: 05 October 2022*
revised manuscript: 15 February 2023
accepted manuscript: 21 April 2023
published online: 12 May 2023
Academic editor; *Korakot Nganvongpanit*

INTRODUCTION

Short DNA fragments (about 200-300 bp) circulate freely in blood plasma and are referred to as cell-free DNA (cfDNA) which are normally present at a low concentration. The cfDNA was first described properly to be present in human blood by Mandel and Metais in 1948. Nearly 50 years later, the blood from a pregnant woman was found to contain cell-free fetal DNA (cffDNA) (Lo et al., 1998). These short DNA can provide valuable genetic information about the fetus or pregnancy. This discovery sparked interest in developing clinical applications based on fetal genetic material analysis for non-invasive prenatal diagnostics. Following that was the indication in plenty of other papers and studies on cffDNA that it may be used to screen for and diagnose fetal genetic disorders during pregnancy (Kelly and Farrimond, 2012; Bunnik et al., 2020; Kjeldsen-Kragh and Hellberg, 2022).

Aside from human research, there are a number of animal experiments aimed at determining fetal sex identity. Jimenez and Tarantal (2003a) examined the amount of cffDNA, the existence of the sex-determining region Y gene (SRY), and the beta-globin gene in the plasma of Gravid Rhesus monkeys (*Macaca mulatta*). The SRY sequences were discovered during the 1st month of pregnancy and the concentrations of cffDNA increased with gestational age and peaked at around the last month of pregnancy from day 130 to 160. Following this work, the research group discovered the existence of fetal DNA in maternal plasma, presenting a new non-invasive method for prenatal gender determination. Serum samples were taken from 72 Gravid Rhesus monkeys between 20 and 32 days of gestation (term 165 ± 10 days) for this investigation. Real-time PCR (RT-PCR) analysis of rhesus Y-chromosomal DNA sequences was used to establish fetal gender and the amount of circulating fetal DNA. By 30 days of pregnancy, the sensitivity for detecting a male fetus was 100% without false-positive results (Jimenez and Tarantal, 2003b). This study shows that maternal serum samples can be used to consistently establish fetal gender in the early first trimester, which is a non-invasive method. Similarly, Yasmin et al. (2015) employed multiplex quantitative RT-PCR to evaluate cffDNA in maternal blood serum taken from 46 pregnant monkeys at gestational weeks 5, 12, and 22. Of 28 monkeys with male-bearing pregnancies, the existence of the SRY gene and DYS14 Y chromosomal sequences was identified. When comparing to postnatal monkeys, the results validated the SRY and DYS14 sequences on the Y chromosome in 28 monkeys, obtaining 100% sex correctness. Since its inception, cffDNA has been used to determine prenatal fetal sex in a variety of mammals, including cattle (Lemos et al., 2011; da Cruz et al., 2012; Ristanic et al., 2018), sheep (Kadivar et al., 2013; Asadpour et al., 2015), mare (de Leon et al., 2012; Tonekaboni et al., 2020), elephant (Vincze et al., 2019) and rhinoceros (Stoops et al., 2018). According to da Cruz et al., (2012) and Wang et al.(2010), polymerase chain reaction (PCR) analysis of fetal DNA in maternal plasma of 35 pregnant calves ranging in age from 5 to 35 weeks allowed them to determine the sex of the fetus at various stages of pregnancy in cows. The fetal sex predicted by this approach 88.6% matched the calf's sex at delivery. Kadivar et al. (2013) used the ovine maternal plasma to screen the SRY gene for fetal sex prediction. Vincze et al. (2019) used two Y-specific markers (AmelY and SRY) to assess fetal sex in elephants. The

presence of these markers suggested a male fetus, while the absence of these markers indicated a female fetus. In particular, this research has ushered in a new era in early gender diagnosis in animals with long gestation periods, with implications for the management of uncommon animal populations. Indeed, cffDNA has been extensively researched and used in both humans and animals. In Vietnam, however, the use of cffDNA in animals has not been conducted. With the purpose of carrying out more research and application in rare and long-pregnant animals, this study initially attempted to detect cffDNA in bovine maternal plasma acquired from farms in the Mekong Delta of Vietnam, and assess fetal sex using a simple, non-invasive method.

MATERIALS AND METHODS

Blood sampling and plasma separation

The experiment was conducted at three dairy cattle farms in Can Tho city, Vietnam. The pregnant crossbred HF and Lai Sind cows (n=11) at different stages of gestation (days 84-235) and non-pregnants (n=2) were chosen for this study. The 8-10 ml blood samples were withdrawn via coccygeal vein using 20G needles and intermediately transferred to BD vacutainer K2E (ref 367525, UK). All samples were stored at 4°C and intermediately transferred to the laboratory. The plasma from the blood samples was extracted by centrifuge at 2,000 rpm for 10 minutes, and then stored at -20°C in the laboratory. The study was conducted with ethical approval for animal care, housing, blood collection under the Animal Welfare Assessments (BQ2019-02/VNCPTCNSH).

Cell free fetal DNA isolation

Cell free fetal DNA (cffDNA) of 13 dairy cows were isolated from the plasma samples using the Plasma/Serum cfc-DNA Purification Midi Kit (Cat No. #55600, Norgen, Canada) according to the manufacturer's instructions. DNA concentration and purity estimation were evaluated spectrophotometrically (Nanodrop ND-2000, Nanodrop Technologies, USA).

Designed-primers used in this study

The 137 bp fragment of the SRY gene was amplified using primer pairs (Table 1) which were self-designed using Primer BLAST software from NCBI according to the Bos taurus, Bos indicus, and Bos taurus x Bos indicus sequence: EU294189.1, XM_19956600.1, KY012736.1, respectively. Similarly, the 109 bp fragment of the GAPDH gene was amplified using a sequence of cattle: NM_001034034.2, XM_027541122.1, and XM_019960295.1, respectively.

Table 1 Sequence of designed primers used in PCR amplification.

Gene	Primers	Sequence (5-3')	Product size
SRY	Forward	GTCTCGTGAACGAAGACGAA	137 bp
	Reverse	GTCTCTGTGCCTCCTCAAAG	
GAPDH	Forward	TCCAAGGAGTAAGGTCCCTG	109 bp
	Reverse	CAGGAGATTCTCAGTGTGGC	

PCR amplification

The C1000 Touch Thermal Cycler (Bio-Rad, USA) was used to conduct the PCR amplifications for the detection of SRY and GAPDH fragments. The reaction volume was 20 (10 µL PCR Master Taq Mix 2X, 0.5 µL forward primer, 0.5 µL reverse primer, 7 µL of nuclease-free water, 2 µL DNA template). The in-house developed amplification was performed using initial denaturation at 94°C for 5 min, followed by 30 cycles with an initial denaturation at 94°C for 40s, the annealing temperature of 58°C for 30s, and extension at 72°C for 30s, followed by a final extension at 72°C for 5 min. The PCR products were loaded on 2.0 % agarose gel by electrophoresis and visualized under UV light. The positive controls were male gDNA samples and negative controls were female gDNA samples. These gDNA were extracted from meat samples.

RESULTS

cffDNA isolation

The results of cffDNA quantify were showed in Table 2. The A260/A280 ratio was used to determine the purity of each extracted cffDNA sample. 'Pure' samples were those with a 260/280 ratio of 1.8 to 2.0. The 260/A280 ratio measurements revealed in this study that the cffDNA samples were of good quality, ranging from 1.8 to 1.9, and could be used in further PCR experiments. Regarding the concentration of cffDNA through the spectrophotometric index, the results showed that cffDNA samples had low concentration (< 200 ng/µL). Notably, the plasma of cow that was 84 days pregnant yielded a DNA concentration of 118,5 ng/µL which value can be applied to PCR reactions as a template. Additionally, these samples of non-pregnant cows with DNA codes 12 and 13 have low DNA concentrations, at 53,7 ng/µL and 50,2 ng/µL, respectively.

Table 2 Concentration and purity estimation were evaluated spectrophotometrically of DNA isolated from maternal plasma

DNA code	1	2	3	4	5	6	7	8	9	10	11	12	13
Acid nucleic (ng/µL)	73.9	116.9	118.5	99.8	80	82.7	89	61.6	87.6	82.2	90.7	53.7	50.2
A260/A280	1.8	1.8	1.8	1.9	1.9	1.8	1.9	1.9	1.9	1.9	1.8	1.8	1.9
Note	P	P	P	P	P	P	P	P	P	P	P	NP	U

P: Pregnant, NP: Non-pregnant, U: Unmarred

PCR amplification of GAPDH and SRY gene fragments

On the acquired cffDNA samples, the SRY and GAPDH genes were amplified. The findings of electrophoresis on 2 % agarose gel demonstrated that the PCR products successfully amplified the SRY and GAPDH genes on cffDNA (Figure 1 and 2). The band size of SRY and GAPDH genes are 137 pb and 109 pb, respectively. All samples including the cffDNA samples and the control gDNA samples were positive for the GAPDH gene as shown in Figure 1 The results Figure 2 revealed that 9 of the 13 lanes (Figure 1, lanes 2, 3, and 5 to 11) were positive for the SRY gene, while 4 samples were negative (Figure 1, lane 1, 4 for 11 of pregnant cows, 12, and 13: cffDNA samples of non-pregnant and unmarred cow, respectively). Nine pregnant cows were expected to produce male calves based on PCR results, whereas two were anticipated to have female calves. The genders of the newborn calves that were recorded at the farm after calving were 100% the same including male and female calves as those predicted by the cffDNA test, as shown in Table 3.

Table 3 Summary of correlations between cffDNA-based PCR pre-determined calves' sex and postnatally determined real sex.

Sex after birth	Numbers of case	SRY gene expression	DNA code
Male	9/11	Positive	2, 3, 5, 6, 7, 8, 9, 10, 11
Female	2/11	Negative	1, 4

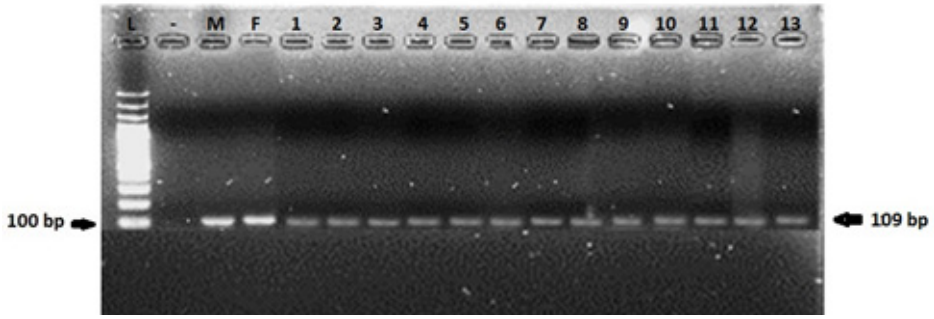

Figure 1 Agarose gel 2% electrophoresis of the GAPDH gene-PCR products from cffDNA, 50V for 30 minutes (L: DNA ladder, (-): Negative control, M: male gDNA sample, F: Female gDNA sample, lane: 1 – 11: cffDNA samples 11 of pregnant cows, 12: cffDNA samples of non-pregnant cow, 13: cffDNA samples of unmarred cow)

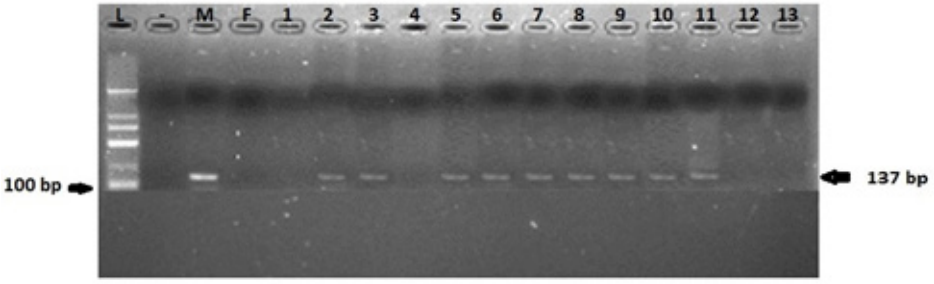

Figure 2 Agarose gel 2% electrophoresis of the SRY gene-PCR products from cffDNA, 50V for 30 minutes. (L: DNA ladder, (-): Negative control, M: male gDNA sample, F: Female gDNA sample, lane: 1 – 11: cffDNA samples 11 of pregnant cows, 12: cffDNA samples of non-pregnant cows, 13: cffDNA samples of unmarred cows)

DISCUSSION

Cell-free fetal DNA is derived from fetal and/or placental cells that undergo apoptosis, hence the concentration of cffDNA is potentially low. In this investigation, the cffDNA concentration in bovine maternal blood was low. This conclusion was in line with the findings of other investigations (da Cruz et al., 2012; Ershova et al., 2017). Because samples 12 and 13 were from non-pregnant cows, there was a lower concentration than in the other samples, indicating that no cffDNA from a fetal calf was collected. The variation in cffDNA concentration is dependent on several factors, including the stage of pregnancy, health circumstances, and cow species (Wang et al., 2010; Lemos et al., 2011;).

Primary sex determination in mammals is purely chromosomal. The sex-determining region Y gene is responsible for the formation of male phenotypes. According to Koopman et al. (1991), the existence of this Y chromosomal region causes proper male development. The SRY gene was employed as a molecular marker to determine the sex of the bovine fetus in this study. The designed SRY 137 primers were developed to exclusively amplify the 137-bp SRY gene. In animals, this marker was commonly employed to determine fetal sex using cffDNA (Kadivar et al., 2013; Stoops et al., 2018; Vincze et al., 2019).

To minimize false positives caused by the lack of SRY amplification in the female fetus, we employed the GAPDH gene as a housekeeping gene and performed PCR in both male and female bovine gDNA. Negative controls included nuclease-free water samples as well as female gDNA samples. The electrophoresis PCR findings indicated that PCR products amplifying the GAPDH gene showed 15/15 (13 cffDNA samples, 1 female gDNA sample, 1 male gDNA sample) were positive at 109 bp, which is the size of the GAPDH gene. Along with SRY, this housekeeping marker was utilized as an internal control in humans, horses, and cattle (Robinson et al., 2007; Kazachkova et al., 2019; Kadivar et al., 2021).

Crossbreeding between HF cows and indigenous beef breeds results in dairy cows on farms in Vietnam (Nguyen et al., 2022). Even though bovine SRY and GAPDH sequences have been extensively studied globally, the genomic DNA samples we acquired from the farms in the Mekong Delta do not respond to the primer pairs used to detect these two sequences. Thus, we developed primer pairs to identify SRY and GADPH processes for dairy cows and beef cattle grown in our area. This is the first study in bovine SRY, GAPDH sequences, and fetal gender based on cffDNA to be conducted in Vietnam. Animal production, care, nutrition, breeding, and genetics all benefit from fetal gender prediction (Stévant et al., 2018). Furthermore, using cffDNA for prenatal sex diagnosis is a simple and reliable procedure. Hundreds of samples can be analyzed quickly by taking blood from cows, rendering this diagnosis appropriate for intensive dairy farms and helping to overcome the limits of manual or ultrasonic examination methods. CffDNA also creates the potential for study and applications in the early detection of genetic abnormalities in cattle and animals with long gestation periods.

One limitation of the study is the relatively small sample size, which may limit the generalizability of the findings. Additionally, the study only focused on the use of cffDNA for fetal sex determination and did not investigate the use of cffDNA for genetic disease screening. However, the strengths of the study include its non-invasive approach to fetal sex determination and the successful isolation of cffDNA in the blood of pregnant cows in Vietnam. These findings suggest that cffDNA-based sex determination could be a reliable and practical method for cattle farmers and veterinarians in the Mekong Delta region and beyond.

CONCLUSIONS

High-purity cell-free fetal DNA could be isolated from the maternal peripheral blood of pregnant cows at different stages of gestation (days 84 - 235). With universal designed primers, the results of the PCR product on the SRY gene and the housekeeping gene GAPDH gene exhibited obvious bands on both gDNA and cffDNA. The results match the actual sex results of calves after delivery (9/13 male calves, 4/13 female calves). It is concluded from this study that cffDNA extracted from the blood of pregnant cows can accurately determine fetal sex using PCR.

ACKNOWLEDGEMENTS

This research was supported by funding from the Scientific Research Fund of Can Tho University, project code: TSV2019-139.

AUTHOR CONTRIBUTIONS

This work was conducted with contribution of all authors. TTTK, NDK, NVL, and CTTN designed the experimental procedures. TTTK, NVL, TVBN, and TGH performed the experiments. TTTK, NTN and LPT interpreted the data and prepared the manuscript. All authors read and approved the final manuscript

CONFLICT OF INTEREST

Regarding the publishing of this article, the authors declare that there are no conflicts of interest.

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How to cite this article;

Khuong, T.T.T., Khoa, N.D., Loi, N.V., Nam, T.V.B., Huy, T.G., Ngu, N.T., Thanh, L.P., Nguyen, C.T.T. Cattle fetal sex determination using cell-free fetal DNA from maternal blood in the Mekong Delta, Vietnam. Veterinary Integrative Sciences. 2023; 21(3): 657- 665.