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Bovine prenatal sex determination using cell free fetal nucleic acid present in maternal plasma

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ABSTRACT

Circulating Nucleic acids (CNAs) are extracellular or cell free nucleic acids which circulate in the bloodstream of healthy pregnant animals. The presence of CNAs in maternal plasma offers the non-invasive prenatal diagnoses of pregnancy associated disorders and also gives opportunities to detect prenatal sex using circulating fetal Y chromosome specific nucleic acid sequence. The present study was done to detect any significant difference in the level of CNAs in the plasma of pregnant (n=27) and non-pregnant (n=3) and recently calved (n= 11) Indian cross bred cows on different gestation stage. Plasma CNAs concentration ranged between 3 to 9.4 ng/µl in pregnant cows which was significantly higher ($p<0.001$) than non-pregnant cows (0.54 ng/µl). PCR amplification of SRY and AMEL gene were used to detect fetal sex using cfDNA. qRT-PCR assay was also used to confirm the presence of fetal RNA in the maternal blood based on SRY gene. PCR based prenatal fetal sex determination showed 80 per cent sensitivity, 100 per cent specificity and 88.89 percent accuracy, whereas qRT-PCR based assay showed 93.33 percent, 100 per cent and 96.29 per cent sensitivity, specificity and accuracy respectively.

Keywords : Cell free nucleic acid, prenatal sex determination, SRY, AMEL.

INTRODUCTION

The discovery of CNAs in the circulatory system has opened up the interesting new area of investigation and new possibilities for molecular diagnosis in various conditions. The existence of CNAs in the plasma of human beings was first reported by Mandel and Metais in 1948 (Fleischhacker and Schmidt 2007). CNAs enter plasma by various ways such as spontaneous release of DNA/RNA lipoprotein complexes, apoptosis and necrosis of nucleated cells, release of cell-derived exosome membrane vesicles, etc. These CNAs play an important role in cell to cell communication, signalling, and horizontal DNA transfer, etc., (Mayer *et al.*, 2012). A healthy individual has very less concentration of cell free DNA (cfDNA) in the plasma or serum. But, Swarup and Rajeswari (2007) reported that the cfDNA concentration is increased during disease conditions such as cancer, stroke, trauma, myocardial infarction, autoimmune disorders, and pregnancy-associated complications.

The mechanism of fetal cell transfer to maternal blood was well known in humans and rhesus monkeys. This is due to the haemochorial placenta enabling fetal DNA transfer to occur at an early stage during the implantation of the conceptus at around day 8-10 (Mayer *et al.*, 2012). In human, fetal DNA in maternal plasma comprised a mean of 3.4 per cent and 6.2 per cent ss of total DNA in early and late gestation,

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respectively (Lo *et al*., 1998) and it cleared rapidly immediately after birth. Previous research conform the presence of cell free fetal nucleic acids in maternal plasma of livestock species. In case of ovine (Kadivar *et al*., 2013) and in equine (Leon *et al*., 2012) PCR base *SRY* gene used to prenatal sex determination and agree the presence of fetal CNAs in the maternal plasma. Cows have synepitheliochorial type of placenta which shows no direct contact between the trophoblast and the maternal blood. The implantation of the embryo occurs after 18 days of gestation. The mechanism for the passage of DNA to maternal plasma still remains unclear in cattle (Lemos *et al.*, 2011).

In cattle, identification of fetal sex at an early stage can change the value of the pregnancy and it gives the chances to plan for better breeding. It also helps in diagnosis of genetic diseases of cattle during the pregnancy. Hence, Fetal nucleic acids are used as tool for early pregnancy diagnosis and fetal sex determination in cattle.

Aim of the present study was to use a PCR assay to detect cfDNA and quantify the amount of cfDNA in the blood plasma of pregnant cows and establish a simple and accurate method to determine fetal sex based on *SRY* gene. Also use it as method of pregnancy diagnosis. Quantification of the fetal RNA and fetal DNA present in maternal blood plasma using real-time PCR was done to determine the early pregnancy in cows.

MATERIAL AND METHODS

Sample collection and preparation

In the present study, a total of 42 healthy animals (male 1, pregnant 27, non-pregnant 3 and recently calved cows 11) maintained at various organized farms were included. Cows from first, second and third trimester of pregnancy was included in the study. Approximately 2 ml of blood was obtained from the jugular vein puncture using the sterile EDTA coated polypropylene tubes. The samples were immediately processed for the plasma separation as per the method of Davoudi *et al.* (2012). To separate the maternal plasma, blood was centrifuged at 1000 rpm for 10 minutes paused and then centrifuged at 1200 rpm for 10 minutes. The supernatant was collected without disturbing the buffy coat and RBC. It was again centrifuged at 2000 rpm for 5 minutes to remove cell debris. The supernatant (plasma) was separated in a sterile labelled 2 mL microfuge tube and stored at -80 ^{o}C until further processing for DNA extraction. To isolate RNA, isolation 350 µl of plasma sample was mixed with 650 µl Trizol reagent and then stored at -80 ^{o}C until further processing.

Cell free nucleic acid extraction from plasma

Cell-free fetal DNA (cfDNA) was extracted from the maternal plasma using QIAamp ® DNA Mini kit (50) and cell free RNA (cfRNA) was isolated from maternal plasma using Trizol chloroform method. The purity and concentration of isolated cfDNA and cfRNA was checked by using Picodrop (Picodrop Ltd, Cambridge, UK). The RNA samples were immediately processed for cDNA synthesis by using Thermoscientific Revert Aid First Strand cDNA Synthesis Kit.

PCR amplification of Bovine AMEL gene / SRY gene

PCR was carried out using the published primers to detect Y chromosome specific sequence present in CNAs as shown in Table-1. The standard PCR was performed in a final volume of 25 µl containing 10 pmol of each primer, 10 µl of DNA sample and 12.5 µl of Ampliqon Taq DNA Polymerase Master Mix red (2X). For each sample, two reactions were performed, one with *AMEL* and another with *SRY*. One male positive control was included in each run. An aliquot of each reaction was subjected to electrophoresis in 2 per cent agarose gel.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

The relative quantification of the *SRY* gene concentration was determined in bovine blood plasma at different stages of pregnancy. Real time qRT-PCR amplification were performed on Eppendorf Master

Table-1. List of primers used for PCR and qRT-PCR analysis

Gene	Primer sequence	Product size	Annealing	Reference	Used in
Bovine *AMEL*	Forward: 5'-GGCCAACACTCCATGACTCCA-3' Reverse: 5'-TGGGGAATATYGGAGGCAGAG -3'	241 bp (X) 178 bp (Y)	60^{o}C 45 sec	Gokulakrishnan *et al*. (2012)	PCR
Bovine *SRY*	Forward: 5'-CCAATTAAGCCGGTCACAGT -3' Reverse: 5'-GCACAAGAAAGTCCAGGCTC -3'	162 bp (Y)	56^{o}C 30 sec	Hamilton *et al*. (2009)	PCR and qRT-PCR
GAPDH	Forward: 5'-GCATCGTGGAGGGACTTATGA -3' Reverse: 5'-GGGCCATCCACAGTCTTCTG -3'	67 bp	56^{o}C 30 sec	Bahar *et al.* (2007)	qRT-PCR

cycler Realplex with Fast Start Universal 2x SYBR Green Master (Applied Bio System Cat No: 4309155) in a 20µL reaction. The nucleotide primer sequences for *SRY* and GAPDH as given in Table-1 was used for the qRT-PCR quantification. Reactions were set in 20 µl volume, using 4 µl of cDNA template, 4 µl of quantitative PCR master mix and 5 pmol of each primer. Cycling conditions were an initial denaturation of 94^0 C for 40 secs, annealing temperature of 56^0C for 30 secs and an extension of 72^0C for 32 secs. qRT-PCR reactions were duplicated for all samples. A no-template control (NTC), female control (FC) and male control (MC) were included in each run. The fluorescence signals were recorded continuously during the temperature ramp. CT values were recorded for the analysis. Accuracy, sensitivity and specificity of molecular sex determination were calculated.

Statistical Analysis

Comparisons of cfDNA concentration was made between different gestational periods (first, second and third trimester) and with calved cows (one day after calving), non-pregnant cows, by one-way ANOVA using SAS system.

RESULTS

cfDNA Concentration

The extracted plasma cfDNA concentration was quantified using Picodrop (Picodrop Ltd, Cambridge, UK) and from that value, the concentration of cfDNA / µl plasma was deducted. The results are prorated in Table-2 and the statistical analysis showed (Figure-1) the significant difference in cfDNA concentration at various stages of gestation. The concentration of plasma cfDNA increases with increase in gestational period (Kadivar *et al*., 2013).

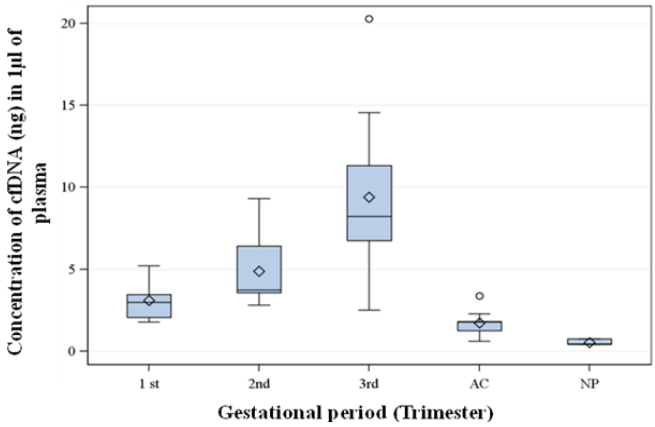
Table-2: Cell free DNA concentration (ng/µl) of plasma in pregnant and non-pregnant cows

Different Stages in Cattle	Number of samples	Mean ± SE
Overall	41	4.78 ± 0.66
1st Trimester	6	3.09 ± 0.50^a
2nd Trimester	9	4.88 ± 0.74^a
3rd Trimester	12	9.40 ± 1.32^b
One day After calving	11	1.73 ± 0.22^c
Non Pregnant	3	0.54 ± 0.10^d

Mean value with different superscripts are different significantly at p<0.001

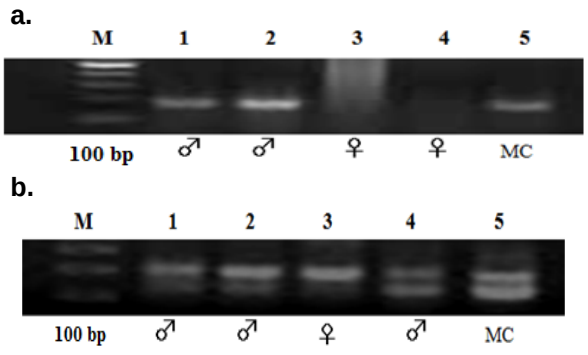
During pregnancy, the concentration of cfDNA in plasma ranges from 3 to 9.4 ng/µl. The maximum cfDNA concentration 9.04 ng/µl of plasma was found during third trimester which was significantly (p<0.001) higher than that of early trimesters, recently calved and non-pregnant animals. The concentration was found to be reduced significantly one day after calving (1.73 ng/µl) while the non-pregnant cows showed significantly (p<0.001) very low plasma cfDNA concentration (0.54 ng/µl) when compared to pregnant cows. But, there was no significant difference between first and second trimester.

Figure-1. Distribution of cell free fetal DNA concentration (ng) per µl of maternal plasma



In the present study fetal sex was predicted based on Y specific *AMEL* and *SRY* gene amplification using bovine blood plasma cfDNA. In male, the amplified gene has yielded two different types of PCR product at 241bp (X Chromosome)and 178bp (Y Chromosome), Where as in female only single PCR product at 241bp. Similarly the *SRY* PCR using the cfDNA from blood plasma of bovines with male fetus showed a single band at 162bp whereas no amplification of the cfDNA from bovines with female fetus was observed.

Figure-2: Amplified PCR product in 2.5% agarose gel electrophoresis. (a) Y specific *SRY* gene amplicon (162bp). (b) *AMEL* gene amplicon shows X chromosome (241bp) and Y chromosome (178bp). M-marker, MC-male control, ♀- female fetus, ♂-male fetus.



Thus the presence of single band in *SRY* and double band in *AMEL* amplicon corresponds to male foetus while absence of band in *SRY* and single band in *AMEL* amplicon corresponds to the female fetus. These PCR

Figure 3. Real-time PCR amplification plot for *GAPDH* and *SRY* gene in cfRNA of pregnant cows

A. Expression profile of GAPDH to conform the presence of cell free plasma nucleic acid.
B. 3rd trimester pregnant cattle samples were used for sexing of fetous by Y chromosome specific *SRY* gene expression.
C. *SRY* gene expression during 1st, 2nd and 3rd trimester of pregnant cattle carries male fetous, shows difference in cfRNA concentration. MC male control, FC female control, ♀ female fetus, ♂ male fetus.

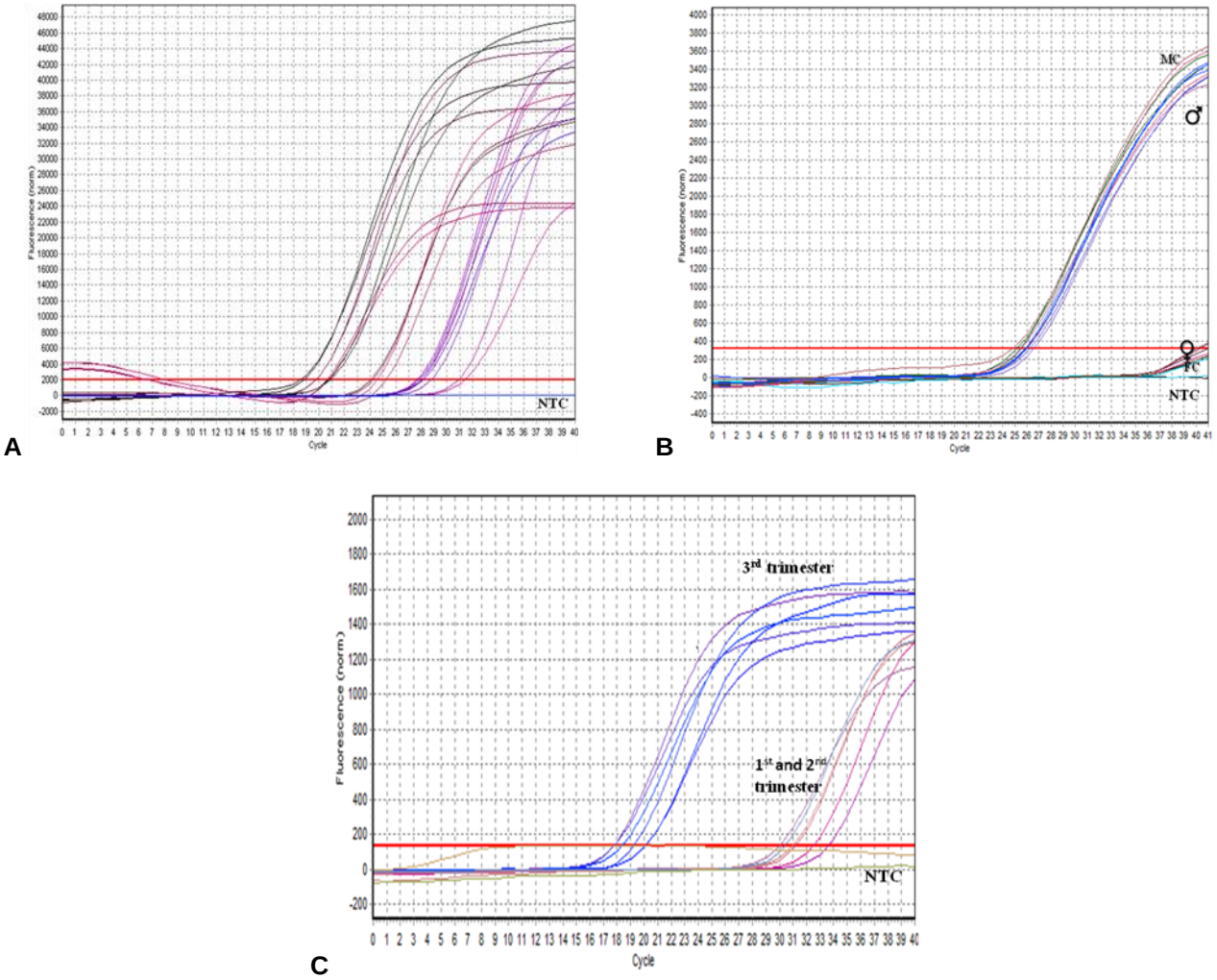


Table. 3: Result of fetal sex finding by using PCR and qRT-PCR analysis of maternal plasma cell free nucleic acids

Stage of gestation	No of samples	PCR result		qRT-PCR result		Actual result found after calving	
		Male	Female	Male	Female	Male	Female
1st Trimester	6	2	4	3	3	4	2
2nd Trimester	9	2	7	3	6	3	6
3rd Trimester	12	8	4	8	4	8	4
Sensitivity 80 per cent (PCR) 93.33 per cent (qRT-PCR)				Specificity 100 per cent (PCR) 100 per cent (qRT-PCR)		Accuracy 88.89 per cent (PCR) 96.29 per cent (qRT-PCR)	

results were verified with calf sex after birth. The results are illustrated in Table-3. The PCR based amplification of *AMEL* and *SRY* gene for sex determination had 80 per cent sensitivity, 100 per cent specificity and 88.89 per cent accuracy.

The PCR based sex determination depending on Y chromosome amplification in plasma sample of the cows bearing male foetus was also studied by Cruz *et al.,* (2012) and Davoudi *et al.* (2012). Equine fetal sex determination using PCR based *SRY* gene in cfDNA

resulted in a sensitivity of 72 per cent for male foetus and an overall accuracy of 85 per cent (Leon *et al.,* 2012). In human prenatal fetal sex was detected using nested PCR for amelogenin gene resulted in 100 per cent sensitivity and 93.8 per cent reliability (Zhu *et al.,* 2005). Another study in ovine *SRY* gene for sex determination showed a sensitivity and specificity of 100 per cent to identification of all male and female foetuses correctly (Kadivar *et al*., 2013). The sex specific amplification of *AMEL* and *SRY* gene from cfDNA technique is relatively simple, fast, inexpensive, and very sensitive to detect and amplify specific genome regions (Cruz *et al*., 2012).

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

This study was performed to confirm the presence of fetal RNA in maternal plasma by real time PCR analysis of GAPDH and *SRY*, based on the research of Poon *et al*, (2000), who reported that cfRNA is also found in the maternal blood. There was amplification in all the samples for *GAPDH*, which demonstrates 100 per cent efficiency of cDNA conversion. The male control (MC) showed amplification for *SRY* gene and no amplification was found in female control (FC). The florescence signal observed in the test sample with curve similar to male positive control indicated male fetus and the test sample without amplification similar to FC indicated female fetus. The results are illustrated in the Table-3 and Figure-3. The qRT-PCR technique for fetal sex determination showed 93.33 per cent sensitivity, 100 per cent specificity and 96.29 per cent accuracy. Prenatal sex prediction in equine by *SRY*/qPCR had 90.9 per cent sensitivity and 95 per cent accuracy (Leon *et al*., 2012). Another, scientist determined the sex of the cattle fetus using maternal plasma cfRNA by ZRSR2Y gene based qRT-PCR assay and their results showed 82.6 per cent accuracy, 75 per cent sensitivity and 100 per cent specificity (Lee *et al.,* 2013). qRT-PCR improves the sensitivity, specificity and accuracy than the conventional PCR assay. Florescent signalling (Dissociation curve) for *SRY* gene amplification in male fetus bearing cow sample showed difference between early and advance pregnancy, CT value of the 3rd trimester samples ranged from 19-21 and the 1st and 2nd trimester ranged from 25-31. This difference may be due to lower concentration of cell free RNA in the 1st and 2nd trimester of pregnancy (Figure-3C). Both PCR and qRT-PCR assay showed similar results. The absence of Y-chromosome in female helps to differentiate the fetal sex. The absence of Y-chromosome gene in maternal plasma showed that the fetus was female. Some of the sample result showed as female because of undetectable concentrations of cfDNA in the presence of male fetus (Wright *et al.,* 2009).

Y chromosome specific gene expression in maternal plasma suggests that the transfer of fetal derived cell to the maternal system. The mechanism behind the leakage of fetal nucleic acid to the maternal circulation is not completely understood. Cell free nucleic acids in the maternal plasma act as biomarker and give an opportunity to the researcher to work towards early pregnancy diagnosis by using the cell free nucleic acids. There is a need to increase adequate number of productive livestock to produce more animal protein to meet out requirements of the growing human population. So, livestock industry has major concern to improve the reproductive efficiency of the animals. Early pregnancy diagnosis is also important as it shortens the calving interval of the animal. Dairy farmers need to diagnose pregnancy at the early state to reduce the management cost and also provide appropriate treatment in case of infertility

CONCLUSION

In conclusion, the present study reveals the possibility of using cell free nucleic acid present in maternal plasma to achieve early pregnancy diagnosis and a cost effective non-invasive method to determine fetal sex through PCR and qRT-PCR techniques.

Conflict of Interests

Authors declare that there is no conflict of interests regarding the publication of this paper.

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