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Early Diagnosis of 60, XX/XY Chimerism in Cattle using RT-PCR

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ABSTRACT

Background: Freemartinism is a special kind of cell chimerism occuring in female calf born co-twin with male calf. It is one of the most common forms of intersexuality found in cattle, arises as a consequence of vascular anastamosis between fetuses of different genders. Freemartinism causes alterations in the development of female reproductive organs and sterility. However, there were reports of fertile heterosexual twins also. Hence, the present study aimed at early diagnosis of 60, XX/XY chimerism in heterosexual pregnancies to prevent the unnecessary economic losses and preserve valuable genetic material.

Methods: Chromosomal profile of female and male calves were analyzed through short term lymphocyte culture technique. The gene expression levels of *ZFX* and *SRY* genes were determined through SYBR green real-time PCR.

Result: Both female and male co-twins were found to possess 60, XX/60, XY chimerism, yielding the PCR products of both the male (*SRY*) and female (*ZFX*) specific genes. A real-time PCR revealed higher levels of *ZFX* gene expression in female (81.66 per cent) and male (88.35 per cent) calves than *SRY* gene. Combination of karyotyping and real-time PCR gave a confirmative diagnosis of freemartinism, suggesting the early diagnosis soon after birth.

Key words: 60/XX, Cattle, Karyotyping, Real-time PCR, XY chimerism.

INTRODUCTION

One of the most common kinds of intersexuality found in cattle is freemartinism (Padula, 2005; Di Meo *et al*., 2008; Peretti *et al*., 2008), a sterile female calf, born with a male co-twin healthy fetus, with rudimentary or undeveloped genitalia (Esteves *et al*., 2012). Usually, freemartinism occurs in early stages of development due to formation of vascular anastamosis between heterosexual fetuses, which ultimately leads to disrupted differentiation of female gonads and genital tract in further developmental stages (Kozubska-Sobocinska *et al*., 2016, Padula, 2005 ; Schlafer and Miller, 2007). The male co-twin shows only minimal defects with slight reduction in fertility (Padula, 2005).

Earlier reports, revealed that 82 to 97 per cent of female calves born co-twin with males showed 60, XX/60, XY leukocyte chimerism (Slota *et al*., 2004; Padula, 2005; Esteves *et al*., 2012; Qiu *et al*., 2018), which, will be sold for meat or production of fetal bovine serum (Hirayama *et al*., 2007). However, there were reports of fertile female co-twins (Qiu *et al*., 2018; Bierman *et al*., 2010 and Szczerbal *et al*., 2014). Hence, early diagnosis of female calves of heterosexual pregnancies would prevent dispensable economic loss and preserve valuable genetic material (Biswas *et al*., 2015). Hence, the objective of the present study was to investigate the suitability of female and male co-twins for further breeding, at early age by screening through cytogenetic and molecular genetic techniques.

MATERIALS AND METHODS

Clinical case

The samples were collected from the Large animal gynecology ward, Madras Veterinary College, Chennai. The work has been carried out between august, 2019 to December, 2019. Heterosexual twin calves (10 days) born to an indigenous (non-descript) cow were presented with female calf having abnormal external genetalia with long coarse of hairs at ventral commissure (Fig 1). Blood samples were collected from both male and female calves in sodium heparin vials and transported to laboratory at 4°C for cytogenetic and molecular studies.

Cytogenetic analysis

Chromosomes were analyzed using short term lymphocyte culture technique standardized in Cytogenetics Laboratory, Madras Veterinary College, Chennai (Harshini *et al*., 2020). A minimum of 200 metaphase spreads per animal were examined to estimate the proportion of 60, XX and 60, XY cells.

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Molecular analysis

Genomic DNA was isolated using standard phenol-chloroform extraction procedure (Sambrook *et al*., 1989) with slight modifications by using DNAzol reagent (Invitrogen, USA), instead of sodium dodecyl sulphate (SDS) and proteinase K. The *ZFX* and *SRY* genes were selected respectively as X- and Y-chromosome specific genes and PCR was carried out with primers described by Tan *et al*. (2015).

Conventional PCR was carried out with an optimized 10 µl reaction containing 5 µl of 2 × PCR master mix (Amplicon), 10 picomoles/ µl of 1 µl each of forward and reverse primer, 50 ng/ µl of 1 µl template DNA and 2 µl of nuclease free water (NFW). PCR conditions were 5 min of initial denaturation at 95°C, followed by 35 cycles of 94°C for 30 sec (denaturation), 62°C for 30 sec (annealing) and 72°C for 30 sec (extension) and 10 min of final extension at 72°C. PCR amplificons were verified by using horizontal submarine gel electrophoresis with 2 per cent w/v agarose, visualized under UV illuminator (Bio-Rad Laboratories Inc., USA) and images were stored. Specific gene amplification was identified by comparing the size of the product with 100 bp DNA ladder marker.

In addition, real-time PCR was carried out in Bio-Rad CFX96 system with amplification profile of initial denaturation at 95°C for 1 min, denaturation at 94°C for 30 sec, annealing at 62°C for 30 sec, extension at 72°C for 30 sec and melt curve at 72-98°C every 0.2°C/sec. The reaction mixture (10 µl) contained 5 µl of SYBR green master mix (TB Green), 1 µl (10 picomoles) of forward primer, 1 µl (10 picomoles) of reverse primer, 1 µl (50 ng) of sample DNA and reaction volume made up to 10 µl with nuclease free water (NFW). Standard curves for *ZFX* and *SRY* genes were generated using purified PCR products ranging from 1×10^6 to 1×10^2 copy numbers. Copy numbers of each gene calculated separately and converted into percentages by using the formula given by Parati *et al*. (2006).

RESULTS AND DISCUSSION

Chromosomal analysis of female and male calves, born co-twin, to an indigenous cow sired by a Jersey crossbred revealed leukocyte chimerism of 60, XX/60, XY on the basis of giemsa staining and morphology of the sex chromosomes (Fig 2). Out of 200 spreads screened, 107 (53.45 per cent) spreads were showing 60, XX cell line, while the remaining (46.55 per cent) spreads were showing 60, XY cell line in the female calf. On the other hand, 109 (54.5 per cent) spreads were found to possess 60, XX cell line and 91 (45.5 per cent) with 60, XY cell line, out of 200 spreads analyzed in the male calf (Table 1).

Though, cytogenetic analysis is the traditional method, it is still effectively used to diagnose the freemartin condition. Diagnosis by cytogenetic testing was proved to be 95-99 per cent accurate when 100 metaphase spreads were studied (Dunn and Johnson,1972; Mc Niel *et al*., 2006). The present case was also confirmed through counting 200 metaphase spreads and was further confirmed by molecular diagnostic tools by screening for X- and Y-specific genes and comparing the results with healthy animals.

Molecular analysis through PCR amplification of *ZFX* and *SRY* genes revealed the presence of both the genes in female and male calves upon 2 per cent agarose gel electrophoresis (Fig 3). In further, real-time PCR analysis carried out with SYBR green master mix resulted in identification of absolute proportion of *ZFX* and *SRY* genes presented in both the co-twins (Fig 4). The expression levels (in percentage) of *ZFX* and *SRY* genes in freemartin and healthy calves are given in Table 1. In the freemartin calves, the expression of *ZFX* gene was found to be higher (*i.e*., 81.66 and 88.35 per cent in female and male co-twins respectively). While, the per cent expression of *ZFX* gene was 100 per cent in a normal female and 50.75 per cent in a healthy male calf.

The conventional PCR results revealing the presence of both *ZFX* and *SRY* genes in male and female co-twins corroborated with the earlier reports (Pourjafar *et al*., 2012; Demyda-Peyras *et al*., 2014; Kozubska-Sobocinska *et al*., 2019), as evident from the sizes of the PCR products. The identification of *SRY* gene with real-time PCR gave even more accurate results as observed by Qiu *et al*. (2018) and Kozubska-Sobocinska *et al*. (2019). In the present study, the relative content of *ZFX* and *SRY* genes in female



Fig 1: (a) An indigenous cow with its twin calves; (b) Female co-twin having tuft of hair at ventral commissure.

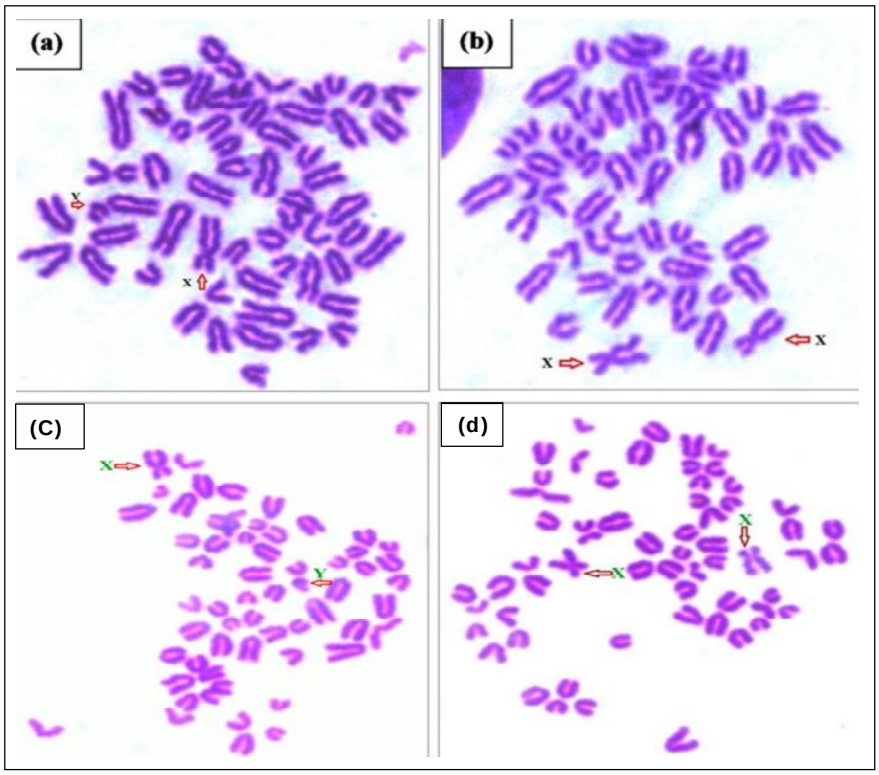

Fig 2: Metaphase spreads showing 60, XX/60, XY chimerism in a female calf (a and b); male calf (c and d) (born co-twin).



Lanes 1 and 2 - *ZFX* gene amplification (475 bp); Lane 3 - No template control for *ZFX* gene; Lanes 4 and 5 - *SRY* gene amplification (525 bp); Lane 6 - No template control for *SRY* gene; Lane 7 - 50 bp Marker; Lanes 1 and 4 - Freemartin female co-twin; Lanes 2 and 5 - Freemartin male co-twin.

Fig 3: Agarose (2%) gel electrophoresis showing amplicons of *ZFX* and *SRY* genes in heterosexual twins.

Table 1: Comparison of *ZFX* and *SRY* gene expression between a freemartin co-twins and normal calves.

Animal	60, XX metaphase spreads (in per cent)	60, XY metaphase spreads (in per cent)	*ZFX* gene (in per cent)	*SRY* gene (in per cent)	Remarks
Freemartin calf	53.4	46.6	81.66	18.34	Higher *ZFX* gene expression
Male co-twin	54.5	45.5	88.35	11.65	Higher *ZFX* gene expression
Healthy female*	100	-	100	-	Normal
Healthy male*	-	100	50.75	49.25	Normal

* - calves born separately (single born) to different dams.

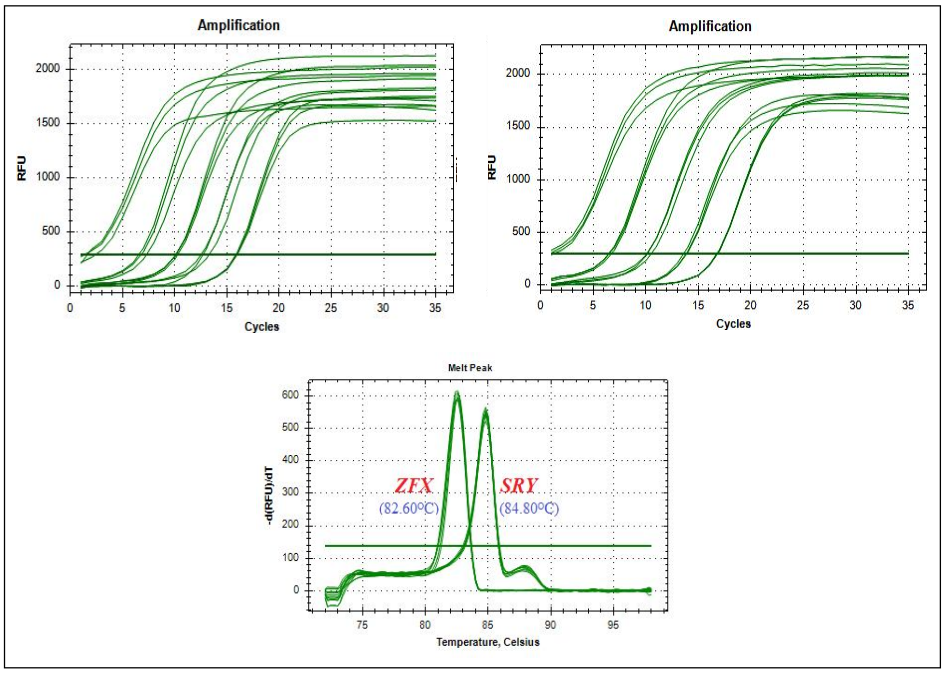

Fig 4: (a) *ZFX* amplification curve, (b) *SRY* gene amplification curve and (c) Melt curve of *ZFX* and *SRY* genes showing single product amplification.

(81.66 and 18.34% respectively) and male co-twin (88.35 and 11.65% respectively) revealed the high expression levels of *ZFX* gene compared to normal male and female calves. Unlike conventional PCR, SYBR green assay gave the definitive and differential gene expression levels of *ZFX* and *SRY* genes, which additionally supported the leukocyte chimerism of freemartin and male co-twin identified through cytogenetic screening. Dunn *et al*. (1979) and Padula (2005) reported that presence of minimal defetcts with slightly reduced fertility in male co-twins compared to female co-twin heifer.

Freemartin condition incurs an indirect loss to the dairy industry and it is a not an hindrance to genetic progress. Diagnosis of freemartin calves and male co-twins is necessary immediately after birth to save the farmer's time and amount spent towards the maintenance of these unproductive animals. The diagnosis of the condition based on clinical signs such as tuft of hair on ventral commissure, short length of vagina, abnormally developed clitoris or external genetalia is not possible, as many of the signs may not manifest immediately after birth. In present study, the male co-twin showed 60, XX/60, XY chimerism, but assessment of fertility could not be done at the early age. Numerous studies had demonstrated that leukocyte chimerism in males associated with lower fertility parameters, reduced non-return rates, lower sperm concentration and motility and also higher degenerative alterations in testicular structures (Cribiu and Popescu, 1982; Switonski *et al*., 1991). The reason attributed to the biopotential nature of primordial-gonadal cells of fetus, that it could differentiate into either male or female (Ottolenghi *et al*., 2007; Norling *et al*., 2013; Piprek *et al*., 2017). The presence of *SRY* gene initiates testicular differentiation via up-regulation of *SOX9* gene. As well as lower levels of SRY in it alics gene expression causes inadeqaute SOX9 in it alics gene levels, which would be deficit to initiate testicular differentiation (Rebourcet *et al*., 2014; Ryan, 2014). Once the expression of *SOX9* gene reaches to optimum level, the biopotential-gonadal cells differentiate into leydig and sertoli cells (Shoemaker *et al*., 2007; Benko *et al*., 2011). However, in the present case, both the calves showed low *SRY* gene expression compared to normal animals and even then, one fetus had become differentiated into a female and other as male. This may be due to formation of anastamosis between the fetuses after the period of initial differentiation of reproductive organ (Szczerbal *et al*., 2014).

CONCLUSION

The cytogenetic analysis, besides, being successfully utilized to diagnose the freemartin condition, the Real-Time qPCR gave a confirmative diagnosis through distinguishing the functionality of *ZFX* and *SRY* genes.

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Conflict of interest

All authors declare that they have no conflict of interest.

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