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Research Article

# A Rapid Multiplex PCR Method for the Diagnose of Freemartin Syndrome in Domestic Cattle (*Bos taurus*)

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ABSTRACT

The majority of heifer calves born as hetero–sexual twin births are sterile freemartins. It is important that this condition be diagnosed at an early age because freemartin heifers can't be used as replacement stock. Different methods are available for the diagnosis of freemartinism, however molecular methods are preferred due to their accuracy and shorter duration of process. In the present study, freemartinism status of 40 female calves of heterosexual multiple births was investigated by multiplex polymerase chain reaction (multiplex PCR). The reaction was carried out using two primer sets for the Sry and *K*–casein genes of domestic cattle. The PCR product was analyzed by agarose gel (3%) electrophoresis, which allowed to identify the genotypes of the animals studied: normal males (453 bp and 163 bp), normal females (453 bp), and 39 (97.50%) study cases that showed chromosome chimerism (freemartin, 453 bp and 163 bp). Compared to the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) technique, this method (i.e., multiplex PCR) proved a far cheaper and quicker (approximately 27 minutes) way to diagnose freemartinism.

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## INTRODUCTION

The term *freemartin* is used to refer a sterile heifer (*farrow* = infertile animal and *mart* = heifer), and is by extension, used to refer to a genetic female from a heterosexual multiple birth. In over 90% of such cases, chorionic vascular anastomoses connecting the placentas of the twin fetuses occur at 30 to 40 days of gestation, i.e., before sexual dimorphism takes place. This results in an interchange of cells (hematopoietic chimerism) and plasma substances such as hormones between the twin embryos, leading to an intersexual state in the female (Harvey, 1976; Gustavsson and Johansson, 1980; Long, 1990). This intersexual condition is characterized by external genitalia that are essentially female in appearance, and internal genitalia that are affected to varying degrees, typically involving gonadal hypoplasia, inhibition of the Müllerian ducts, masculinization of the gonads, and stimulation of the Wolffian ducts (Tran et al., 1977; Edwards et al., 1994; Harikae et al., 2012).

The incidence of freemartin syndrome in heterosexual bovine twins has been estimated to be 92% (McFeely et al.,, 1967; Marcum 1974; David et al., 1976). However, freemartins can also occur in single female births, as a result of the early fetal death and reabsorption of the male twin within the uterus after the development of vascular anastomoses and once sexual differentiation has occurred (Smith et al., 1977). The basic techniques traditionally used to diagnose freemartin syndrome have included clinical examination, tolerance to homo–grafts test, blood typing, and cytogenetic analysis. However, the advent of the polymerase chain reaction (PCR) technique and the use of specific DNA sequences of the bovine Y chromosome constitute significant advances in the detection of freemartins, particularly when few of the calf's cells have an XY chromosome complement. Compared to the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analysis, the multiplex polymerase chain reaction (multiplex PCR) method proved a far cheaper (not using restriction enzyme) and quicker (no time restriction) way to diagnose freemartinism (Olsaker et al.,. 1993; Ennis et al.,. 1999; Padula, 2005).

## MATERIAL AND METHODS

### *Animals and Samples*

A total of 40 *Bos taurus* calves (31 Holstein, 4 American Brown Swiss, 2 Jersey, and 3 Simmental), all products of multiple heterosexual births, were selected to be tested for freemartin syndrome. The clinical history of each animal

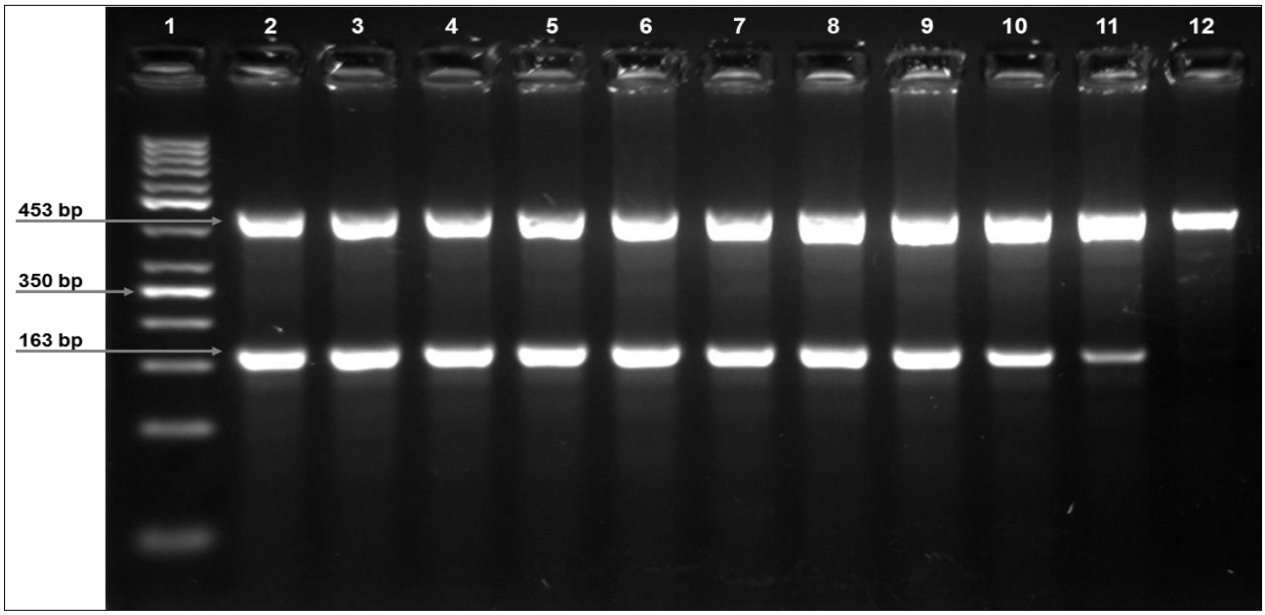


Figure 1: Agarose gel electrophoresis of male bovine DNA amplified by PCR to determine the optimal annealing temperature of the primers (Sry and *K*–casein) using the gradient PCR program; Lane 1, molecular marker (50 bp Fermentas®); lanes 2 – 12, DNA samples for each of the various annealing temperatures (°C) of the program

was taken and blood sample was collected for molecular analysis by jugular venipuncture using a vacutainer needle and vacuum tubes containing 1.5 mg/mL of EDTA.

***DNA Extraction***

DNA was isolated from the blood samples of each animal; 100 µL of blood was mixed with 900 µL of buffer A (0.32 M sucrose; 10 mM Tris HCl; 5 mM $MgCl_2$; 1% Triton X–100). The mixture was then centrifuged at 12,000 rpm for 2 minutes and the supernatant then removed; this process was repeated until a white cell pellet was formed. Next, the sample was incubated for one hour at 50ºC in a Proteinase K solution (8 mg/mL) in buffer D (50 mM KCl; 10 mM Tris–HCl; 2.5 mM $MgCl_2$; 0.455 NP–4O, 0.45% Tween–20). The Proteinase K was inactivated by incubating the sample at 90ºC for 10 minutes (Ayala–Valdovinos et al., 2007).

***Gradient PCR***

Gradient PCR, using the nucleotide sequence of the Sry gene (GenBank accession number NM_001046429.2) and *K*–casein gene (GenBank accession number BC102120.1) of bovine, was performed. For each gene were employed two previously reported oligo–nucleotide primers (5´–TGAACGCTTTCATTGTGTGGTC–3´ and R 5´–GCCAGTAGTCTCTGTGCCTCCT–3´ for the Sry–F gene (Pomp et al., 1995); and F 5´–TGTGCTGAGTAGGTATCCTAGTTATGG–3´ and R 5´–GCGTTGTCTTCTTTGATGTCTCCTTAG–3´ for the *K*–casein gene (Barroso et al., 1997); followed by a standard PCR protocol (final volume 20 µL), with 100 *n*g of DNA, 0.50 U of Taq DNA Polymerase, 0.2 mM of dNTPs, 0.4 µM of each primer, 1X of PCR buffer, 1.5 mM of $MgCl_2$, and 10.55 µL of $ddH_2O$. The amplification cycles were performed in a gradient thermal cycler (TC–5000, Techne, USA) with 40 cycles of denaturation at 94°C for 60 seconds, the annealing temperature having been previously determined by the equipment's gradient program; the extension temperature was 72°C for 60 seconds.

Subsequently, the amplification product was analyzed by GelRed–stained agarose gel electrophoresis with a 1X TBE buffer solution (Rickwood and Hames, 1990). Lastly, the DNA bands were observed and photographed under UV light in a gel documentation system (Kodak Gel Logic 200®).

***Multiplex PCR***

Based on the gradient PCR, was selected the annealing temperature (54°C) for performing multiplex PCR with the chosen Sry and *K*–casein gene primers. For a fast multiplex PCR protocol, a high–speed thermal cycler (Thermo Scientific Piko, Finland) and 0.2 mL ultra–thin–walled PCR tubes (Thermo Scientific®) were used as follows: 95°C for 2 minutes, followed by 32 amplification cycles at 94°C for 5 seconds, 54°C for 5 seconds, 72°C for 15 seconds, and a final extension of 72°C for 3 minutes.

## RESULTS

The optimal annealing temperature of the oligo–nucleotide primer used in this study (Sry and *K*–casein) was determined by using the gradient PCR program (Techne gradient thermal cycler, UK). Figure 1 shows the electrophoretic run of 11 samples containing DNA from the same male bovine control specimen, which were amplified at a different annealing temperature in accordance with the equipment's program (lane 2, 50.2°C; lane 3, 50.8°C; lane 4, 52.3°C; lane 5, 54°C; lane 6, 55.5°C; lane 7, 57.2°C; lane 8, 58.8°C; lane 9, 60.7°C; lane 10, 62.2°C; lane 11, 63.9°C; lane 12, 65°C). A wide range of optimal annealing temperatures for the primers were found (lanes 2 – 9). Based on these experiments, the annealing temperature selected for this pair of primers was 54°C (lane 5). With the primers used in this study, through the dilution of male bovine blood in the female bovine blood, it was shown that XY cells were detectable up to a concentration of 0.1%.

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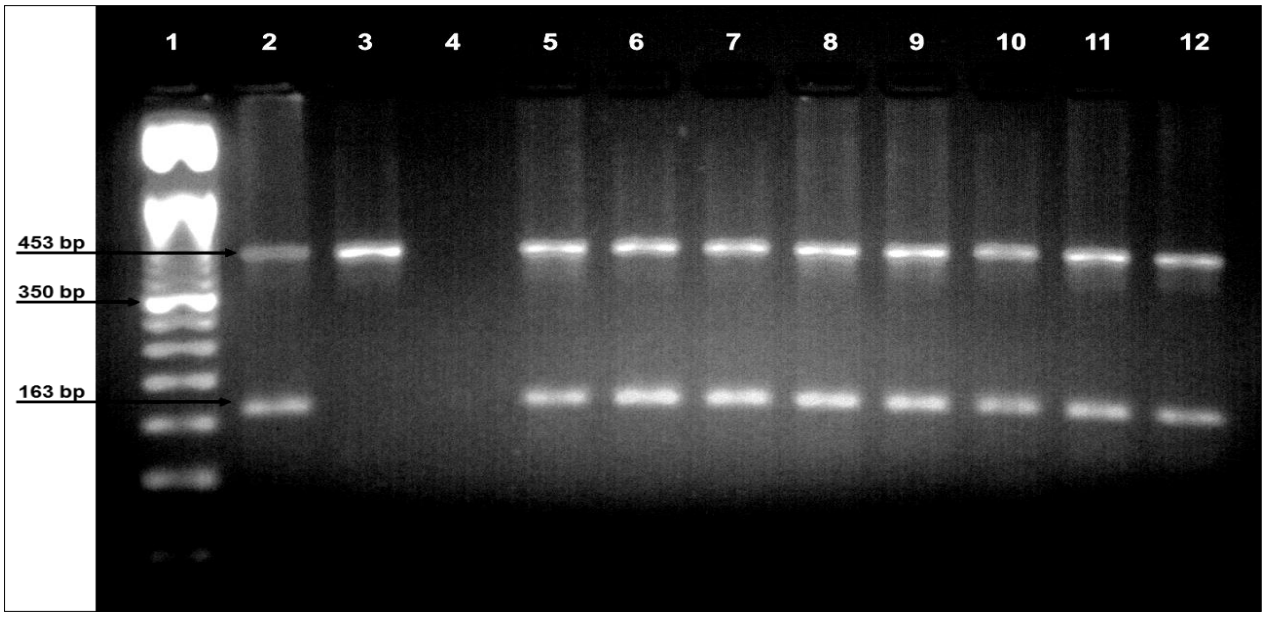


Figure 2: Agarose gel electrophoretic analysis of multiplex PCR of different samples of bovine DNA with amplification of the Sry (163 bp) and *K*–casein genes (453 bp); Lane 1, molecular marker (50 bp Life Technologies®); lane 2, male control; lane 3, female control; lane 4, negative control; lanes 5 to 12, freemartin females

The fast multiplex PCR method taking approximately 27 minutes allowed the amplification of the genomic DNA fragments corresponding to the Sry (163 bp) and *K*–casein (453 bp) genes of the cattle

The product of the multiplex PCR was analyzed by agarose gel (3%) electrophoresis stained with ethidium bromide, allowing to identify the genotypes of the animals tested: normal males (453 bp and 163 bp), normal females (453 bp), and freemartins (453 bp and 163 bp) (Figure 2), which resulted in the diagnosis of 39 (97.50%) cases of intersex conditions due to freemartin syndrome, in a sample of 40 heifers born of heterosexual multiple births.

## DISCUSSION

Freemartins display certain clinical signs, particularly in terms of their genitalia, though the appearance of the external genitalia of the newborn calf is sometimes relatively normal. In certain cases, a freemartin calf displays elongation of the clitoris and a tassel of hair on the lower commissure of the vulva; however, these features are inconsistent and not particularly reliable for diagnostic purposes (Long, 1990; Padula, 2005; Sohn, 2007).

The homograft tolerance test is a complicated procedure and the male twin is not always available, therefore laboratory testing is necessary. The cytogenetic analysis for diagnosing freemartinism identifies the possible chimeric condition (XX/XY) in either heterosexual twin. Ideally, however, both twins should be sampled given their tendency to exhibit similar proportions of cells. In cases where XX/XY chimerism has occurred with relatively low frequency of XY cells, it is important to examine many metaphase spreads to be certain of the diagnosis of freemartinism. Statistically, 90 XX cells must be counted before we can be 99% certain that no XY cells are present in the female (Harvey, 1976; Long, 1990; Padula, 2005). Furthermore, poor–quality blood samples for the culture of lymphocytes prevent the collection of suitable metaphases for the cytogenetic analysis of freemartin syndrome (McNiel et al., 2006).

Male–specific DNA amplification methods, based on polymerase chain reaction (PCR), have particularly been used for sex chromosomal chimerism analysis in peripheral leukocytes because of their high sensitivity and rapidity (Hirayama et al., 2007). The present study demonstrates that the use of multiplex PCR is more efficient, less expensive, and more practical for routine early diagnosis of the bovine freemartins.

This study allowed us to identify 39 (97.50%) freemartin heifers using a fast multiplex PCR technique involving the use of oligonucleotide primers for the Sry and *K*–casein genes, which clearly underlines the high frequency of the syndrome reported in the literature in cases of multiple heterosexual births in this species.

## CONCLUSION

Molecular diagnostics (PCR–RFLP or Multiplex PCR) provides a useful tool with which to reach an accurate and timely diagnosis of the most commonly found intersex condition in domestic cattle, that is, freemartin syndrome, thereby making it possible to identify animals that would have no reproductive or productive value. Compared to the PCR–RFLP technique, this multiplex PCR technique offers a far cheaper and quicker (i.e., around 27 minutes) means of diagnosing freemartinism.

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