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LEGITIMACY OF DIAGNOSIS OF 60,XX/60,XY CELL CHIMERISM IN CALVES BORN FROM MULTIPLE DIFFERENT-SEX PREGNANCIES

Anna Kozubska-Sobocińska, Grzegorz Smołucha

National Research Institute of Animal Production, Department of Molecular Biology of Animals, 32-083 Balice near Krakow

Abstract

On the basis of many years of research in cattle, it is assumed that the phenomenon of multiple pregnancies affects about 2% of births, while 82-95% or even 97% of heifers from different-sex twin pregnancies have leukocytic chimerism (60.XX/60.XY) and phrymartinism, with the remaining 3-18% of females developing normally. Taking into account the fact that the frequency of multiple pregnancies in dairy cattle has recently increased significantly, in some breeds up to 20%, it can be assumed that the number of fertile twins has also increased. This trend was also confirmed by cytomolecular studies of HF heifers born with a male twin, the results of which allowed to state that 12.5% of such heifers do not show phrimartinism and can be qualified for reproduction. For this reason, early and accurate identification of females with a karyotype of 60.XX and born from multiple different-sex pregnancies is extremely important, as it prevents the elimination of valuable female material from breeding. Given these arguments and the increasing need for rapid diagnosis of phrimartinism, cytomolecular studies of multiparous calves are needed to provide a basis for accurate guidelines and selection recommendations, limiting the financial outlay caused by keeping animals unsuitable for breeding.

Keywords: chimerism 60,XX/60,XY, phrimartinism, cytomolecular diagnostics, Polish Holstein-Friesian cattle

Introduction

The phenomenon of cellular chimerism refers to the occurrence in a given individual of two or more cell lines with different genetic assumptions. XX/XY chimerism, occurring during fetal development, is the result of implantation into the embryo of cells originating from the mother or, in the case of a multiple pregnancy, from a co-twin. Chimerism may also arise from the fusion of zygotes, and may also be the result of simultaneous fertilisation by two sperm of the egg cell and the corpus callosum (Ford, 1969; Jankowski and Ildstad, 1997).

In cattle, cellular chimerism is most often observed in individuals from twin or multiple heterozygotic pregnancies, either single or different sexes. The presence of several cell lines is the result of the formation of common bloodstream between developing embryos and the exchange of hematopoietic tissue through anastomoses, that is, vascular connections between the fetal membranes of co-twins (Kastli, 1974; Khan and Foley, 1994).

Effect of cellular chimerism 60,XX/60,XY on carrier fertility

On the basis of many years of research carried out on different cattle populations, it is assumed that 82-95% of heifers from different-sex multiple pregnancies are carriers of leukocytic chimerism and associated phrimartinism (Kozubska-Sobocińska et al., 2019). Phrymartins, i.e. females with a karyotype of 60,XX/60,XY, are sterile, and sterility is caused by extensive pathological changes in the reproductive system. The external genitalia in most cases are typically female, while the most frequently recorded lesions include an enlarged clitoris, a small, often blind-ended vagina, hypo- and even aplastic uterus (Khan and Foley, 1994; Zhang et al., 1994; Peretti et al., 2008). Hypoplastic, sometimes masculinised gonads contain ovarian-nuclear structures and, in a few cases, structures resembling a tumour developing in the monolayer epithelium (Padula, 2005; Kozubska-Sobocińska et al., 2011). It should be emphasized that the percentage of XY cell line, borrowed from the male twin, does not determine the degree of masculinization of the reproductive organs of phrymartin, as evidenced by abnormal internal genitals often found with a small percentage of male cells (Nowacka et al., 2004; Padula, 2005; Esteves et al., 2012; Kozubska-Sobocińska et al., 2016). It is also worth mentioning that cytogenetic studies often diagnose cases of cell chimerism in heifers registered in breeding documentation as derived from single births, when the male co-twin dies in the early fetal period (Esteves et al., 2012; Szczerbal et al., 2014; Iannuzzi

et al., 2021). There has been a long-standing discussion on the effect of the presence of females during embryonic development on the subsequent breeding suitability of males derived from twins or multiples of different sexes (Kozubska-Sobocińska and Rejduch, 2008; Kozubska-Sobocińska et al., 2016). Numerous studies of bovine twins have shown that bulls with a leukocyte chimerism of 60.XX/60.XY are most often characterised by parameters of fertility, semen quality and suitability for freezing that do not deviate from generally accepted norms and such bulls can therefore be conditionally allowed to breed (Gustavsson, 1977; Kovács and Karakas, 1997). In contrast, a number of other reports have suggested a reduction in fertility rates in such bulls, particularly oestrus uniqueness index and semen parameters (De Giovanni et al, 1975; Cribiu and Popescu, 1982; Świtoński et al, 1991). The assessment of semen quality, based on studies of ejaculates obtained from bulls with a karyotype 60, XX/60,XY, showed a significant or highly significant decrease in ejaculate volume, sperm motility and concentration, and a higher percentage of damaged sperm with major and subordinate morphological defects, as well as a higher incidence of degenerative changes in the testicular structure, sometimes azoospermia and weak libido (Dunn et al., 1979; Rejduch et al., 2011).

Diagnosis of cellular chimerism

Conventional method of karyotype assessment

Cytogenetic studies of farm animals are aimed at identifying chromosomal aberrations and karyotype defects, which generally have adverse effects on fertility and the development of animals. A special type of karyotype abnormality is leukocyte chimerism, expressed by the formula 2n,XX/2n,XY, the diagnosis of which depends on the identification of cell lines differing in a set of heterosomes.

This abnormality can be easily diagnosed in the karyotype of cattle. Due to the specific chromosome morphology in this species: twenty-nine pairs of acroccentric autosomes and characteristic X and Y heterosomes (meta- or submetacentrics), it is possible to identify 60,XX/60,XY chimerisms in a light microscope, directly on chromosome preparations, stained routinely with Giemsa dye (photo 1) (Dunn et al., 1979; Long, 1990; Słota et al., 2000; 2004).

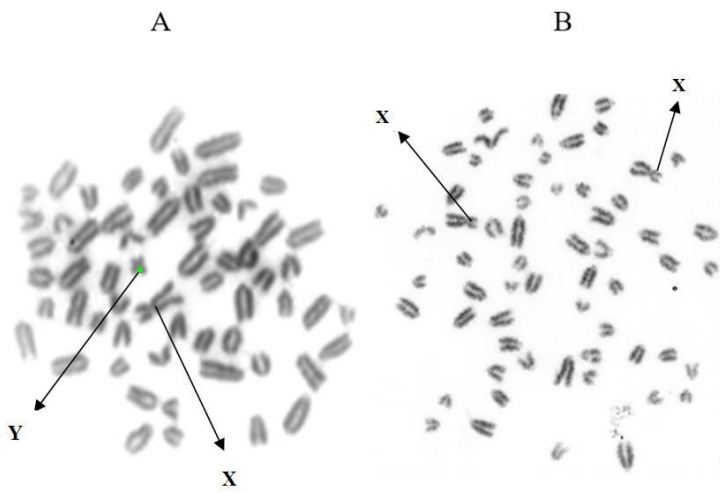

Photo 1. Metaphase chromosomes of a heifer with 60,XX/60,XY karyotype: cell lines 60,XX (A) and 60,XY (B)

For other species belonging to the *Bovidae*, such as sheep and goats, in which cellular chimerism occurs with a similar frequency, the identification of sex chromosomes requires the use of differential staining techniques such as CBG, GTG, RBA and QFQ, the most useful of which is the CBG technique, as it allows the acrocentric X heterosome to be distinguished from autosomal chromosomes, due to its characteristic feature, which is a weakly colored C-band in the region of centromeric heterochromatin (Iannuzzi and Di Berardino, 2008).

In situ fluorescent hybridization (FISH) technique

Currently, molecular studies of chromosomes using the in situ hybridization *(FISH)* technique are increasingly used in cytogenetic diagnostics. This cytochemic method of hybridization of specific DNA or RNA sequences, labeled with fluorescent dyes, with complementary sections of chromosomomes fixed on a microscope slide, can be used in the diagnosis of small structural mutations, physical gene mapping, and identification of X and Y chromosomes in both somatic and reproductive cells (Słota et al., 2003; Kozubska-Sobocińska et al., 2009; Villagómez et al., 2009; Ron et al., 2011; Barasc et al., 2014; Danielak-Czech et al., 2016).

In the case of possible vascular connections between the fetal membranes of embryos from multisexual multiple pregnancies, the use of the FISH

technique enables precise identification of heterosomes X and Y, which are markers of female and male cell lines (Kozubska-Sobocińska et al., 2003; 2016; Rychlik et al., 2005). Molecular probes used in the diagnosis of XX/XY chimerism are most often probes painting entire sex chromosomes or their fragments, or identifying genes specific to heterosomes (e.g. the SRY gene AMELY, AMELX) (Rejduch et al., 2000; 2004; Revay et al., 2002; Peretti et al., 2008).

Cytomolecular diagnosis of cellular chimerism

In recent years, microsatellite DNA sequence analyses (STR - short tandem repeats) and single nucleotide polymorphism (SNP) analyses, as well as new rapid and precise molecular techniques, have been added to the cytomolecular diagnostic suite using karyotyping and heterosome identification techniques. These techniques include comparative genomic hybridization (CGH), QF-PCR (quantitative fluorescence PCR) based on the analysis of selected STR markers, ddPCR (droplet digital PCR) technique, or Real-Time PCR – a quantitative PCR test with reverse transcription in real time, taking into account sex chromosome-specific STRs (Martinez-Royo et al., 2009; Villagómez et al., 2009; Qiu et al., 2018; Szczerbal et al., 2019).

Important markers used in molecular analyses for the identification of XX and XY cell lines are genes located on heterosomes, particularly the male sex-determining gene SRY and the amelogenin gene occurring in two variants, AMELX and AMELY, specific for the X and Y chromosomes respectively. The differences between the amelogenin gene variants are the presence of a 6 pz deletion in intron 1 of the AMELX gene relative to intron 1 of the AMELY. gene. This deletion is detected by PCR and gel electrophoresis. Two DNA bands indicate two variants of the *AMELX* and *AMELY* gene (XY cell line), while one band indicates the presence of only the *AMELX* gene variant (XX line)(photo 2) (Qiu et al., 2018; Kozubska-Sobocińska et al., 2019).

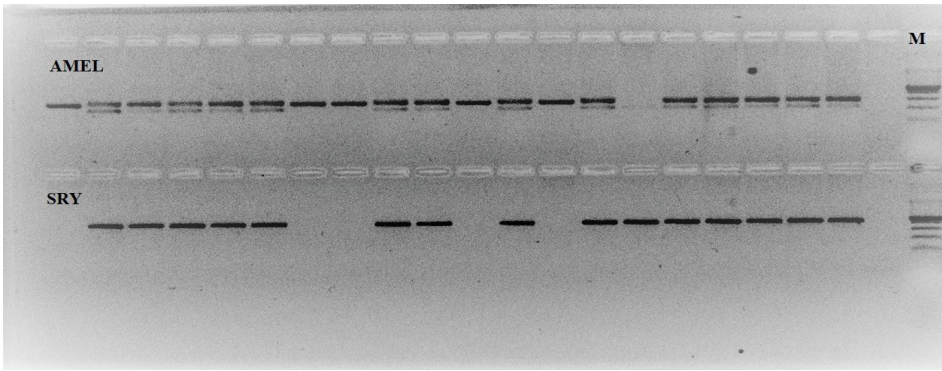

Photo 2. Electrophoretic analysis of bovine DNA samples with amplification of *SRY* (one band visible), and *AMEL* genes; XY – bulls (two bands visible), XX – heifers (one band visible), M – 100 bp DNA marker. No bands means no amplification.

It should be noted that the use of molecular methods makes it possible to identify submicroscopic aberrations, invisible in standard cytogenetic examination, with unprecedented resolution, ranging from several hundred to a dozen or even several base pairs, without the need for cell culture (Mc Niel et al., 2006; Kozubska-Sobocińska et al., 2016; 2019).

Attempts have been made to evaluate the usefulness of cytogenetic and molecular methods for the identification of various cell lines, both in the blood and in other tissues. This is confirmed by studies of blood samples and skin fibroblasts obtained from calves from same- and heterosexual twin pregnancies (Plante et al., 1992; Nowacka et al., 2004; Mc Niel et al., 2006). It was found that only molecular methods, such as microsatellite DNA sequence polymorphism analyses, also make it possible to diagnose cellular chimerism in fraternal same-sex twins. These studies allowed to estimate the reliability and effectiveness of methods for determining cellular chimerism, and also showed that the level of the population of cells received from co-siblings is much higher in the blood than in other tissues (Rejduch et al., 2000; 2004).

Effects of cytomolecular studies on calves born from multiple different-sex pregnancies

Carrier of karyotype abnormalities is usually associated with infertility or reduced fertility, determining the low breeding suitability of breeding animals burdened with these defects.These aberrations cannot be recognized without cytogenetic diagnostics, as they generally occur in individuals with normal exterior (and normal seeding parameters in males) and, through artificial insemination, they can be rapidly spread in populations, causing losses in

economically significant breeding (Yimer and Rosnina, 2014; Raudsepp and Chowdhary, 2016; Danielak-Czech et al., 2020). The improvement of reproductive performance in breeding herds of livestock can be achieved through selection based on early diagnosis and elimination of animals affected by karyotype abnormalities, including leukocytic chimerism, by cytogenetically monitoring young animals in particular before they are used for reproduction. Consequently, many EU countries, including Poland, have systems in place to control the karyotype of breeding stock intended for insemination (Ducos et al., 2008; Kozubska-Sobocińska and Danielak-Czech, 2017). In recent years, the cytogenetic monitoring programme has been intensified, including mandatory cytogenetic testing of all young bulls (before they are destined for reproduction), resulting in the detection of several dozen chromosomal rearrangements and numerous cases of cellular chimerism (Peretti et al., 2008; Ron et al., 2011; Kozubska-Sobocińska et al., 2019; Szczerbal et al., 2019). The results obtained confirmed the effectiveness of the system of karyotype control of insemination sires, justifying the need to continue and intensify cytogenetic screening of breeding cattle in Poland, with a particular focus on young males prior to their use in reproduction, as well as calves born from mixed-sex multiple pregnancies (Kozubska-Sobocińska and Danielak-Czech, 2017; Kozubska-Sobocińska et al., 2019).

In order to emphasize the importance of new cytomolecular methods currently used in the early diagnosis of 60,XX/60,XY cellular chimerism in calves from heterosexual multiple pregnancies, it should be clarified that many years of research of various breeds of cattle have shown that 82–95% and even 97% of heifers from heterosexual twin pregnancies have leukocyte chimerism (60,XX/60,XY) and phrimartinism. In contrast, the remaining 3–18% of females develop normally. Taking into account the fact that the prevalence of multiple pregnancies in dairy cattle has recently increased significantly, in some breeds up to 20%, it can be assumed that the number of fertile twins has also increased (Wiltbank et al., 2006; Bierman et al., 2010). This trend was also confirmed by cytomolecular studies of 24 HF heifers born with a male twin, 3 of which were characterized by the correct karyotype (60, XX). These results allowed to conclude that 12.5% of such heifers do not show phrimartinism and may be qualified for reproduction (Kozubska-Sobocińska et al., 2019). For this reason, it is extremely important to identify early and precise females with a 60,XX karyotype and those born from multiple heterosexual pregnancies, as this prevents the elimination of valuable female material from breeding.

Given these arguments and the growing need for rapid diagnosis of phrimartinism, it is reasonable to seek different combinations of molecular techniques that provide a basis for formulating sound guidelines and selection recommendations.

The elimination from breeding herds of animals burdened with chromosomal anomalies prevents the spread of these genetic defects and also reduces the financial outlay associated with maintaining animals unsuitable for reproduction (Ducos et al., 2008; Danielak-Czech et al., 2020).

It should also be added that the introduction of various combinations of precise and reliable molecular techniques (such as: PCR, STR, Real-Time PCR, ddPCR) for the cell chimerism diagnostic package allows the use of frozen material for testing, greatly simplifying the cumbersome procedure of delivering fresh blood samples to the laboratory within 24 hours of collection (Qiu et al., 2018; Kozubska-Sobocińska et al., 2019; Szczerbal et al., 2019).

The result of early cytomolecular diagnosis of 60,XX/60,XY cell chimerism in calves from multiple heterosexual pregnancies are expert opinions issued to breeders, containing specific indications and selection recommendations regarding the reproductive suitability of carriers of this karyotype abnormality (Kozubska-Sobocińska and Danielak-Czech, 2017; Kozubska-Sobocińska et al., 2019).

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SUMMARY

Based on many years of research in cattle, it is assumed that the phenomenon of multiple pregnancies affects about 2% of births, while 82–95% or even 97% of heifers from different-sex twin pregnancies have leukocyte chimerism (60,XX/60,XY) and

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