A high incidence of leukocyte chimerism (60,XX/60,XY) in single born heifers culled due to underdevelopment of internal reproductive tracts

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ABSTRACT: Freemartinism, a primary disorder of sex development (DSD) in cattle, is associated with leukocyte chimerism (60,XX/60,XY). The diagnosis of DSD is easy if it is known that a heifer with abnormally developed reproductive tracts originates from a heterosexual twin birth, but it is not so obvious in the case of single born calves. In the present study twelve DSD heifers which were single born (singletons) and culled due to the abnormal development of internal genitalia were studied using cytogenetic and molecular techniques. Among the heifers 7 appeared to be chimeric (60,XX/60,XY and the presence of the genes residing in the Y chromosome: *SRY* and *AMELY*) and 5 had a normal female karyotype (60,XX and a lack of the Y-linked genes). In addition, milk productivity was analyzed in relation to the incidence of twinning at a local Dairy Cattle Breeding Centre, from which 8 studied singletons (6 chimeric and 2 with a normal female karyotype) originated. It was found that in the years 2005–2013 an upward trend for average milk yield (from 9700 kg in 2005 to 11 500 kg in 2013) was associated with the increase of twin births (from 1.5% in 2005 to 5.9% in 2013). Our study showed that approximately 60% of single born heifers with abnormally developed internal genitalia were freemartins (a male co-twin died during pregnancy), while DSD etiology of the other cases (60,XX and a lack of the Y-linked genes) remains unknown. It cannot be excluded that some of these heifers represent a testicular/ovotesticular DSD (60,XX and *SRY*-negative). In conclusion, our study suggests that the occurrence of freemartins and other DSD in single born heifers seems to be an underestimated problem in cattle breeding.

Keywords: cattle; disorders of sex development; freemartinism; twinning; intersexuality; milk yield

INTRODUCTION

Decreased fertility of high-producing dairy cattle is a serious breeding problem (Lucy 2001; Wathes et al. 2008). It is known that genetic correlation between milk production and reproductive traits – e.g. days from calving to the first insemination – is quite high and thus unfavourable (Zavadilova and Zink 2013). On the other hand, incidence of twin pregnancies, associated with an occurrence of freemartinism, is also correlated with high milk yield (Kinsel et al. 1998).

Freemartinism is a very well known type of disorders of sex development (DSD) in cattle and other ruminants originating from heterosexual multiple pregnancies (Komisarek and Dorynek 2002; Padula 2005; Estevez et al. 2012). Placental anastomoses between twin fetuses are developed in cattle with a very high incidence (above 90%). In the case of heterosexual twins they facilitate

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migration of masculinization molecules (e.g. the SRY transcription factor, the Mullerian inhibiting substance (MIS), and testosterone) produced by testicles to the female co-twin, which cause an abnormal development of reproductive tracts in the female fetus. It was also suggested that in gonads of freemartins structures resembling neoplasm can also be developed (Kozubska-Sobocinska et al. 2011). An overall incidence of freemartinism in cattle populations depends on the frequency of twin (or multiple) pregnancies. In Holstein-Friesian cattle the twinning rate varies between 4 and 6% and in recent decades an upward trend has been observed (Silva del Rio et al. 2007; Andreu-Vazquez et al. 2012). It clearly shows that freemartinism is an important problem in cattle breeding.

Diagnosis of freemartinism, based on the physical examination of reproductive tracts, can be confirmed by cytogenetic and molecular analyses (Padula 2005; Ron et al. 2011). In freemartins chimerism in blood cells, established due to the development of anastomoses, is usually detected by chromosome studies of leukocytes (60,XX/60,XY) or molecular detection of Y-linked genes (*SRY, AMELY* or *ZFY*). It should be pointed out that the frequency of both cell lines may vary within a very wide range – from 1 to 99% (Nowacka et al. 2004; Perreti et al. 2008). Thus, cytogenetic detection of chimerism may require a laborious analysis of up to several hundred metaphases. On the other hand, detection of the Y-linked genes (*SRY, AMELY* and/or *ZFY*) in blood is a very sensitive and fast technique, but is not sufficient to diagnose XX/XY chimerism.

Physical investigation of heifers, prior to the first insemination, is a common procedure and heifers with abnormally developed reproductive tracts are culled. If such a heifer was born as a co-twin to a male, then the freemartinism condition is assumed, but if the heifer was derived from a single birth, the diagnosis is not easy. It is known that some single born heifers are freemartins, resulting from the death of their male co-twins. Single born freemartins were described for the first time by Wijeratne et al. (1977), who analyzed 5 single born infertile heifers with chromosomally detected leukocyte chimerisms (60,XX/60,XY). To our knowledge, since that time no such cases have been reported. Taking above into consideration, we aimed to perform cytogenetic and molecular analyses of single born heifers, which were culled due to the abnormal development of reproductive tracts.

MATERIAL AND METHODS

Animals. Cytogenetic and molecular analyses were carried out on 12 single born heifers (singletons), culled due to underdevelopment of reproductive traits. Among them 8 heifers originated from a local Cattle Breeding Centre and 4 from different dairy cattle farms. The animals were selected for further cytogenetic and molecular analyses by an experienced veterinarian, who examined the heifers *per rectum* and/or by ultrasound inspection.

Cytogenetic analysis. Chromosome preparations were obtained from a short-term lymphocyte culture. Briefly, heparinized blood was cultured in RPMI 1640 (Sigma-Aldrich, St. Louis, USA) medium, supplemented with fetal calf serum (15%), an antibiotic-antimycotic mixture (1%), and phytohaemagglutinin at 37°C for 72 h. After colcemid treatment a standard harvesting procedure including hypotonic and fixative steps was applied. Chromosome slides were Giemsa stained. For each individual a total of 30 to 100 metaphases were examined. Sex chromosomes were identified based on their bi-armed morphology (X – large submetacentric and Y – small metacentric). Chromosome preparations were analyzed under a Nikon E600 Eclipse microscope (Nikon, Tokyo, Japan).

Molecular analysis. Genomic DNA was isolated from blood, using a commercial kit (A&A Biotechnology, Gdynia, Poland). The presence of three genes (*SRY*, *AMELY,* and *AMELX*) was analyzed. Loci of the *SRY* gene and the *AMELY* pseudogene are present on the Y chromosome, while the *AMELX* gene resides on the X chromosome. To detect the *SRY* gene the following PCR primers were used: F – 5' aagaacaacttatgaatagcacca 3' and R – 5' ttaagtcgcaggtgaaactgt 3'. To detect the *AMELY* and *AMELX,* the same PCR primers were used (F – 5' cagccaaacctccctctgc 3' and R – 5' cccgcttggtcttgtctgttgc 3'), which produce amplicons of different size (two amplicons (280 bp and 217 bp) for males and a single amplicon (280 bp) for females), due to deletion of 63 bp in the *AMELY.* The PCR protocol was as follows: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 s, primers annealing for 30 s at 60°C (for the *SRY* gene) and at 64°C (for *AMELY* and *AMELX*), elongation at 72°C for 40 s, and final elongation at 72°C for 5 min. Amplicons were visualized by agarose-gel electrophoresis.

Table 1. Cytogenetic and molecular analyses of single born DSD heifers

Animal	Karyotype	Distribution of XX and XY metaphases			Molecular detection of genes		
		No. of analyzed metaphases	60,XX (%)	60,XY (%)	*SRY*	*AMELY*	*AMELX*
1	60,XX	30	100	not found	–	–	+
2	60,XX	100	100	not found	–	–	+
3	60,XX/60,XY[a]	50	54	46	+	+	+
4	60,XX/60,XY[a]	50	64	36	+	+	+
5	60,XX/60,XY[a]	50	46	54	+	+	+
6	60,XX/60,XY[a]	50	92	8	+	+	+
7	60,XX/60,XY[a]	50	36	64	+	+	+
8	60,XX/60,XY[a]	100	62	38	+	+	+
9	60,XX	100	100	not found	–	–	+
10	60,XX	100	100	not found	–	–	+
11	60,XX	100	100	not found	–	–	+
12	60,XX/60,XY[a]	80	85	15	+	+	+

DSD = disorder of sex development, +/– = detected/undetected
[a]freemartin

RESULTS AND DISCUSSION

Abnormal development of reproductive organs causes severe economic losses in cattle breeding. From *post mortem* studies, carried out on material collected at abattoirs, it is known that the most frequently observed abnormality are ovarian cysts (Herenda 1987; Kubar and Jalakas 2002; Probo et al. 2011). On the other hand, freemartinism is considered as a primary DSD in cattle (Padula 2005). A highly variable abnormal development of reproductive organs in freemartins is a well-known phenomenon (Peretti et al. 2008; Esteves et al. 2012). However, visible signs of virilization of external genitalia are rarely observed, but if they occur the most common ones include an enlarged clitoris, an increased or reduced ano-vulval distance, the vulva with long coarse hair and a blind-ended vagina. All the twelve studied single born heifers had normal external genitalia and underdeveloped internal genitalia.

Cytogenetic and molecular analyses revealed leukocyte chimerism in 7 heifers (Table 1). The proportion (%) of the XX and XY metaphase spreads varied from 92/8 (case 6) to 36/64 (case 7). Molecular detection of the *SRY* and *AMELY* genes confirmed the chimeric status (Figure 1). Identification of the XX/XY chimerism in singletons is not surprising, since such a possibility was reported earlier (Wijeratne et al. 1977). The authors ana-

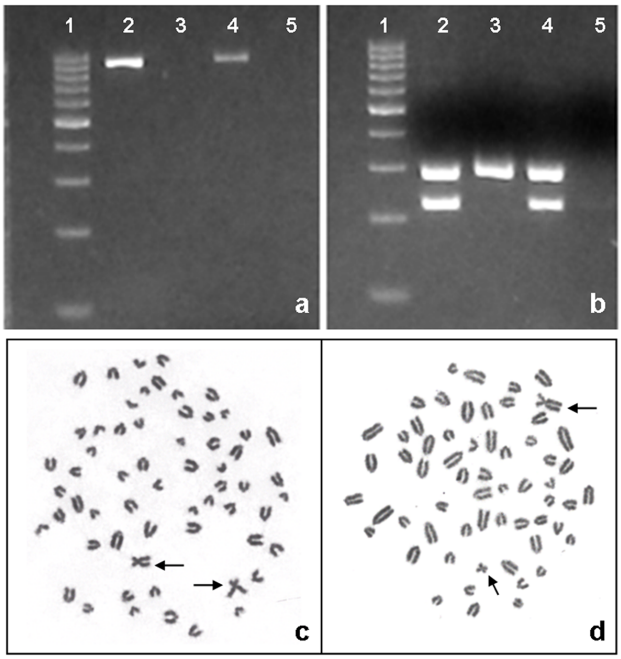

Figure 1. Molecular and cytogenetic studies of heifers with disorder of sex development (DSD)

(**a**) electrophoresis of the *SRY* gene – 851 bp, (**b**) electrophoresis of the *AMELX* and *AMELY* genes. Two bands (280 bp and 217 bp) are detected in males and a single band (280 bp) in females. Lines: 1 = length marker 100 bp Ladder; 2 = reference male, 60,XY; 3 = reference female, 60,XX; 4 = freemartin, 60,XX/60,XY; 5 = negative control. (**c**, **d**) metaphase spreads detected in freemartins: 60,XX (**c**) and 60,XY (**d**). Sex chromosomes are indicated by arrows

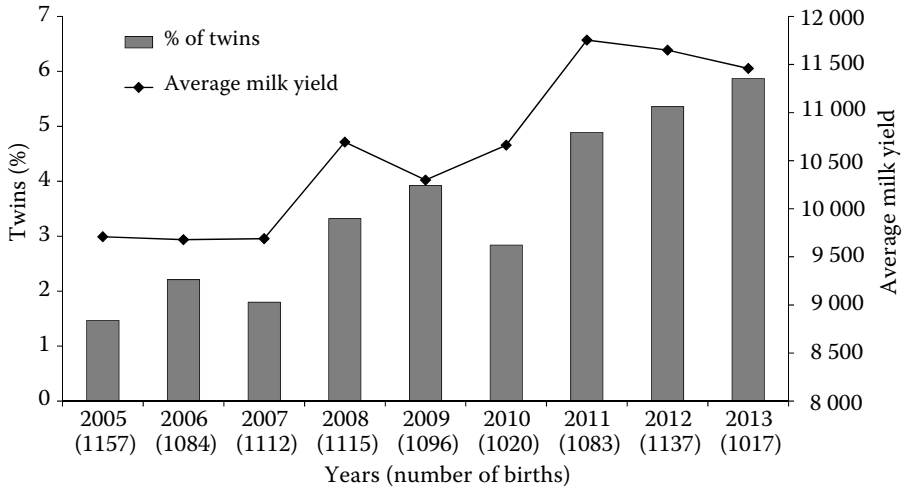

Figure 2. Incidence of twin births and average milk yield at a local Dairy Cattle Breeding Centre in the years 2005–2013

lyzed *post mortem* the development of reproductive tracts in 3 chimeric singletons. Two of them had rather normal reproductive tracts, but their cervix or uterine horns were non-patent. The third one had a normal vagina, an enlarged clitoris, and gonads resembling ovotestes.

Identification of 5 cases with a normal female karyotype (60,XX) and a lack of genes residing on the Y chromosome is intriguing. Based on physical examination, which resulted in the identification of underdeveloped internal reproductive tracts, one can assume that these heifers were freemartins. On the other hand, the lack of chimerism may suggest that during fetal development the placental anastomoses were not developed. There are two possible explanations for this fact. Firstly, it can be hypothesized that in some heterosexual twin pregnancies development of placental anastomoses is followed by migration of masculine substances, but is not accompanied by the settlement of haematopoietic progenitor cells in the bone marrow of the co-twin. In such a situation the disorders characteristic of freemartins are not associated with leukocyte chimerism (60,XX/60,XY). Secondly, it can be assumed that the observed DSDs were caused by other mechanisms. Unfortunately, knowledge on DSDs other than freemartinism in cattle is rather limited. Sex chromosome abnormalities responsible for DSD are not common in this species and X monosomy is almost absent, while cases of XXY, XYY, and XXX trisomies were reported rather rarely (Ducos et al. 2008). There are very few reports on DSD females with a male karyotype (60,XY) and a lack of the *SRY* gene (Kawakura et al. 1996; Payan-Carreira et al. 2008). On the other hand, the hereditary testicular or ovotesticular DSD (the female karyotype and a lack of the *SRY* gene), well-known in goat, pig, horse, and dog, was not reported in cattle (Villagomez et al. 2009). Classification of the testicular or ovotesticular DSD is based on three major criteria: (1) the female karyotype, (2) a lack of the *SRY* gene, and (3) histologically detected testicular or ovotesticular gonads. With regards to external genitalia such animals usually have an enlarged clitoris. All DSD heifers with a normal female chromosome complement (60,XX and a lack of the *SRY* gene) had normally developed external genitalia. Unfortunately, the *post mortem* collection of gonads for histological analyses was not possible. Thus, we can only speculate whether there were cases of the testicular/ovotesticular DSD among these heifers.

Since earlier reports indicated an association between increasing milk yield and the incidence of twinning (Kinsel et al. 1998; Silva del Rio et al. 2007; Andreu-Vazquez et al. 2012) we also looked for such a relationship at the Dairy Cattle Breeding Centre. An upward trend was found for twinning in the analyzed years (2005–2013) (Figure 2). An overall proportion of twin births in this period was 2.91% (calculated for 7667 births), but in the last three years (2011–2013) the ratio was as high as 5%. In the same period an average milk yield also presented an upward trend, from 9708 kg in 2005 to 11 459 kg in 2013. In the last three years the yield exceeded 11 400 kg. These data support results of the reports mentioned above.

CONCLUSION

Our study suggests that freemartinism is an increasing breeding problem, due to an upward trend for twinning rate as well as the occurrence of freemartins among single born heifers. Secondly, we showed that further studies, especially includ-

ing histological evaluation of gonads, should be performed to verify the hypothesis on the occurrence of hereditary testicular/ovotesticular DSD (60,XX; *SRY*-negative) in cattle.

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