The ambiguity of freemartinism diagnosis in cattle revealed by cytogenetic and molecular techniques

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ABSTRACT: Nineteen heifers and three male co-twins, originating from heterosexual twin pregnancies, were analysed with the use of cytogenetic and molecular techniques. A large number (50–400) of Giemsa stained metaphase spreads were studied and the proportion of XX and XY cell lines was calculated. The presence of two genes – *SRY* and *AMEL* (*AMELX* and *AMELY*) – was also analysed. Both approaches facilitated the identification of the XX/XY chimerism in 17 females and three males. The proportion of the co-twin cell line ranged from 1 to 99%. In two females no chimerism was detected: (1) 60,XY male chromosome complement in all 400 metaphase spreads; *SRY*-positive, *AMELY*-positive, *AMELX*-positive and (2) 60,XX female chromosome complement in all 200 metaphase spreads; *SRY*-negative, *AMELY*-negative, *AMELX*-positive. The usefulness of different techniques for the diagnosis of freemartinism is discussed.

Keywords: intersexuality; freemartinism; XX/XY chimerism; cattle

Freemartinism is the most common form of intersexuality in cattle. It concerns a vast majority (over 90%) of females originating from heterosexual twins (for the review see: Komisarek and Dorynek, 2002). The male co-twin to a freemartin is usually fertile and normally developed; however, some cases demonstrating abnormalities of the reproductive organs were also described (Strabel *et al*., 2003). Since freemartinism is responsible for congenital female infertility, its early diagnosis is recommended to avoid economic losses caused by useless therapeutical treatment.

Due to vascular anastomoses developed between the twin foetuses, an exchange of haematopoietic tissue cells takes place and the XX/XY blood cell chimerism in both foetuses is established. The diagnosis of freemartinism is usually based on a routine cytogenetic analysis of lymphocytes, but other techniques (blood typing, blood protein electrophoresis, DNA polymorphisms) also appeared to be powerful in the detection of chimerism. Minisatellite as well as microsatellite DNA polymorphisms were successfully used in the chimerism detection (Plante *et al*., 1992; Rejduch *et al*., 2001). However, it should be pointed out that the above-mentioned molecular techniques are insensitive to distinguish between chimerisms caused by the cell exchange between hetero- or homosexual twins. The analysis of some genes (i.e. *SRY, AMELX/AMELY* or *ZFX/ZXY*) located on sex chromosomes or anonymous markers assigned to the Y chromosome (i.e. BOV97M, BRY.1 and BRY.4a) were also used to diagnose chimerism (Schellander *et al*., 1992; Olsaker *et al*., 1993; Justi *et al*., 1995; Ennis *et al*., 1999). This approach is very adequate to detect the Y chromosome in a heifer, but it does not facilitate to distinguish between the XX/XY chimerism and sex reversal syndrome (female with a male karyotype – 60,XY).

The aim of this study was to compare the cytogenetic and molecular (the identification of the *SRY* and *AMELX/AMELY* genes) techniques in relation to the sensitivity of XX/XY chimerism detection.

MATERIAL AND METHODS

Nineteen heifers originating from heterosexual twins and three full-sib males were included in this study. Blood samples were collected from animals reported by the breeder as a female co-twin to a male. Unfortunately, it was not possible to collect the samples from all co-twin males.

Cytogenetic analyses were performed on Giemsa stained preparations obtained after the 72-hour lymphocyte culture. At least 100 metaphase spreads per animal (with an exception of two animals) were analysed and the proportion of the XX and XY cells was estimated.

DNA was isolated from blood cells with the use of a commercial kit "Blood DNA Prep Plus" (A&A Biotechnology, Gdansk, Poland). A fragment of the *SRY* gene (440 bp) was amplified by PCR using a T-gradient thermocycler (Biometra, Goettingen, Germany). The following primers were applied (GenBank # Z30327):

SRY BD F:
5′ AAGGGGAGAACATGTTAGGGAGAG 3′

SRY BD R:
5′ TTTGCAGGAGTGAATTGGTTATGA 3′

PCR reaction conditions were as follows: 35 cycles denaturation at 94°C for 40 seconds, annealing of primers at 58.6°C for 40 seconds, elongation at 72°C for 1 minute, final elongation at 72°C for 10 minutes. PCR amplification of the *AMEL* gene fragment was carried out with the use of primers described by Ennis and Gallanger (1994):

SE 47: 5′ CAGCCAAACCTCCCTCTGC 3′

SE 48: 5′ CCCGCTTGGTCTTGTCTGTTGC 3′

Two fragments were amplified: 280 bp characteristic of the X chromosome and 217 bp characteris-

Table 1. Cytogenetic and molecular analyses of 22 animals derived from heterosexual twin pregnancies

No.	Sex	Cytogenetic analysis			Molecular analysis	
		number of metaphases	XX (%)	XY (%)	*SRY*	*AMELY*
1[a]	♀	100	81	19	+	+
2[a]	♂	100	84	16	+	+
3[b]	♀	200	99	1	+	+
4[b]	♂	100	2	98	+	+
5	♀	100	66	34	+	+
6[c]	♀	50	52	48	+	+
7[c]	♂	50	38	62	+	+
8	♀	100	24	76	+	+
9	♀	100	59	41	+	+
10	♀	200	2	98	+	+
11	♀	100	28	72	+	+
12	♀	100	97	3	+	+
13	♀	100	12	88	+	+
14	♀	100	8	92	+	+
15	♀	400	0	100	+	+
16	♀	100	14	86	+	+
17	♀	150	99	1	+	+
18	♀	100	37	63	+	+
19	♀	200	100	0	–	–
20	♀	100	50	50	+	+
21	♀	100	30	70	+	+
22	♀	100	4	96	+	+

[a, b, c] twins

tic of the Y chromosome. PCR reaction conditions were similar to those for the *SRY* gene, except the annealing of primers, which took place at 70ºC for 40 seconds. The products of PCR were separated by electrophoresis using 1.5% agarose gel.

RESULTS AND DISCUSSION

Cytogenetic analyses revealed XX/XY leukocyte chimerism in 17 heifers and three males originating from heterosexual twins and the percentage of the co-twin leukocyte cell line ranged from 1 to 99% (Table 1). There was no clear correlation between female and male co-twins concerning the ratio of XX and XY cells. One twin (1 + 2) demonstrated a very similar ratio, but in the other two (3 + 4 and 6 + 7) the proportion was distinctly different in co-twins. In two heifers (No. 15 and 19) no chromosomal chimerism was found in spite of a large number of analysed metaphase spreads (400 and 200, respectively). In the first case (heifer No. 15), a male karyotype (60,XY) as well as both Y-linked genes (*SRY* and *AMELY*) were found. In the second case (heifer No. 19) a female karyotype (60,XX) was identified and the absence of both Y-linked genes was observed. Examples of electrophoregrams of the *SRY* and *AMEL* genes are shown in Figure 1.

The obtained results show that in some cases the detection of leukocyte chimerism in a heifer suspected to be a freemartin is ambiguous. It is well known that the proportion of both cell lines may vary within a very broad range (Komisarek and Dorynek, 2002). Thus, a large number of spreads is recommended for cytogenetic evaluation to detect a cell line occurring with a low frequency. In our

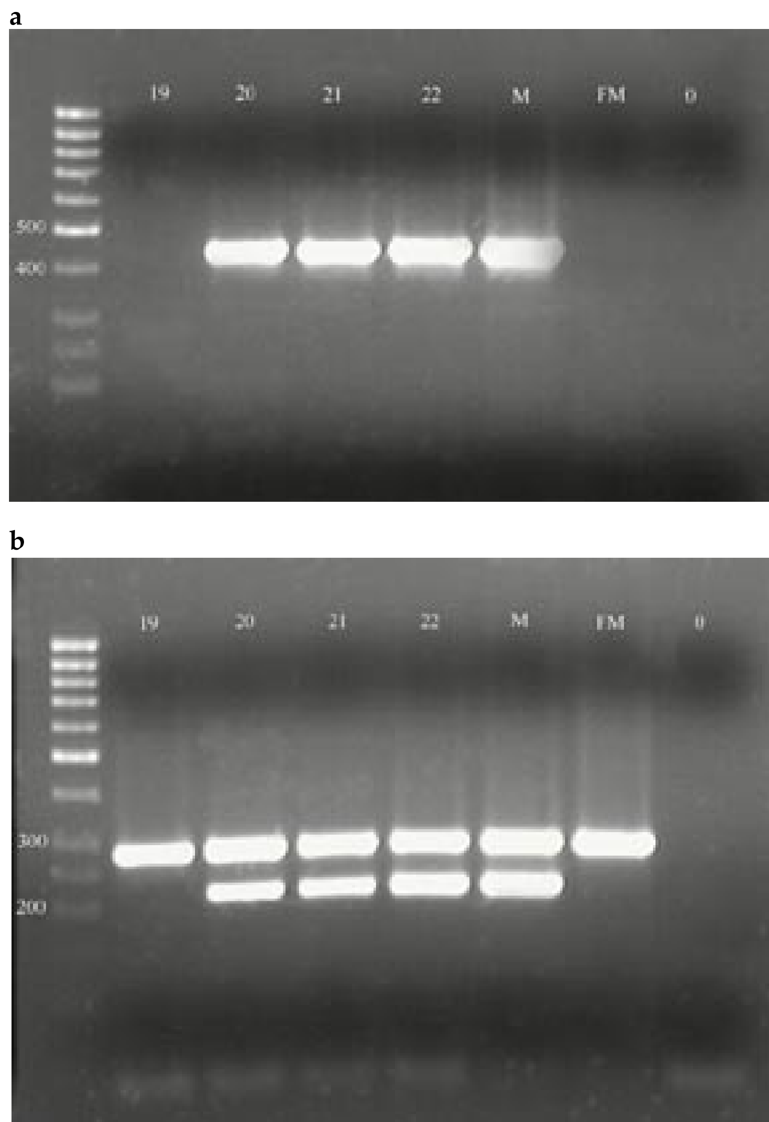

Figure 1. PCR detection of the: (a) *SRY* gene fragment – 440 bp and (b) *AMELX* – 280 bp and *AMELY* – 217 bp gene fragments

M = reference male; FM = reference female; 0 = sample without DNA; 19–22 = the analysed samples (for details see Table 1)
A length marker Gene Ruler^{TM} (50bp Ladder; MBI Fermentas, Vilnius, Lithuania) was used. Note that female # 19 did not exhibit the presence of *SRY* and *AMELY* genes

study one heifer (60,XY, *SRY*-positive, *AMELY*-positive) had the XY sex chromosome complement in all the evaluated four hundred spreads. Molecular analysis revealed the presence of both Y-linked genes: *SRY* and *AMELY*. There are two possible explanations for it: (1) the frequency of its own cell line was extremely low (below 0.25%) or (2) this case represents a sex reversal syndrome not related to freemartinism. The XY sex reversed syndrome (*SRY*-negative) was quite frequently diagnosed in infertile mares (Makinen *et al*., 1999; Bugno *et al*., 2003), but not in cattle. Due to the presence of the Y-linked genes in the heifer one can speculate that it was rather a case of the testicular feminisation syndrome or gonadal dysgenesis. Unfortunately, we were not able to support this suggestion by clinical and endocrinological data (the level of testosterone) and cytogenetic analysis of the bull co-twin. In cattle both syndromes were rarely diagnosed (Popescu, 1990). Another unusual case was heifer No. 19, which had a normal karyotype (60,XX) and did not have any Y-linked genes. This status seems to represent a well-known phenomenon that less than 10% of twin pregnancies do not develop placental anastomoses and consequently, the absence of chimerism is observed. Such a heifer is anticipated to present a normal reproduction performance.

The common use of the microsatellite polymorphism in parentage control aroused an increased interest in the use of this technique for the freemartinism diagnosis (Rejduch *et al*., 2001). It is recommended to analyse DNA isolated from blood cells and hairs with the aim to recognize one's own and full-sib cell lines. Unfortunately, this approach has some important limitations. If the analysis can be performed only on the heifer of interest, the detection of chimerism is not equivalent to the presence of XX/XY chimerism characteristic of freemartinism. It is due to the fact that chimerism may be caused by hetero- or homosexual twin pregnancy. If the Y-linked markers are the only studied ones in DNA isolated from blood cells, then it is impossible to distinguish between XX/XY chimerism and sex-reversal syndrome (female with a male karyotype 60,XY). On the other hand, the analysis of the Y-linked marker polymorphism along with markers localised on autosomes, to study DNA isolated from blood and hair, facilitates the detection of chimerism.

To avoid ambiguities we suggest that the diagnosis of freemartinism should be performed with the use of both cytogenetic and molecular techniques. It is postulated that to detect the XX/XY chimerism at least 100 metaphase spreads should be analysed since the proportion of full-sib lymphocytes may vary within a wide range. The analysis of the amelogenin gene is a simple technique for the identification of both sex chromosomes. A simultaneous application of these approaches is specially recommended for the so-called isolated cases when only a heifer can be analysed. Then, distinguishing between freemartinism and other types of intersexuality, including hereditary ones, is possible.

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ABSTRAKT

Nejednoznačnost diagnostiky freemartinismu u skotu zjištěná pomocí cytogenetických a molekulárních metod

Cytogenetické a molekulární metody byly použity k analýze devatenácti jalovic a tří býčků sourozenců a dvojčat, kteří pocházeli z heterosexuálních březostí se dvěma plody. Byl vyšetřen velký počet (50–400) Giemsou barvených metafází a vypočítán podíl buněčných linií XX a XY. Rovněž byla provedena analýza na přítomnost dvou genů – *SRY* a *AMEL* (*AMELX* a *AMELY*). Oba přístupy vedly k identifikaci chimérismu XX/XY u 17 jaloviček a tří býčků. Podíl buněčné linie dvojčete-sourozence se pohyboval od 1 do 99 %. U dvou jaloviček nebyl zjištěn žádný chimérismus: (1) 60,XY samčí chromozómový komplement ve všech ze 400 metafázových roztěrů; pozitivní *SRY*, *AMELY* i *AMELX* a (2) 60,XX samičí chromozómový komplement ve všech ze 200 metafázových roztěrů; negativní *SRY* a *AMELY*, pozitivní *AMELX*. V článku se diskutuje vhodnost různých metod diagnostiky freemartinismu.

Klíčová slova: intersexualita; freemartinismus; chimérismus XX/XY; skot

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