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CYTOGENETIC AND MOLECULAR DIAGNOSTICS OF XX/XY CHIMERISM IN CATTLE, SHEEP, AND GOATS – A REVIEW

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Abstract

Animal cell chimerism denotes the presence of two or more cell lines of different genetic origin. The most common form of chimerism, which occurs spontaneously in mammals, is the presence of two or more cell populations in animals derived from dizygotic twin or multiple pregnancies. The aim of this review is to present extensive information on XX/XY chimerism in cattle, sheep and goats from the *Bovidae* family. Additionally, it will discuss a methodology for a quick and accurate diagnosis of cell chimerism and make an analysis of cells obtained from gonads of animals derived from twin pregnancies which underwent an exchange of hematopoietic tissue as a result of anastomoses. The studies reviewed here led the authors to conclude that XX/XY chimerism is generally undesirable in livestock breeding. Females (freemartins) are infertile, whereas in male carriers of chimerism, the impact of this abnormality on sperm parameters is still under discussion, therefore every case of different-sex twin or multiple births should be analyzed individually.

Key words: *Bovidae,* XX/XY chimerism, freemartinism, cytomolecular diagnostics

Characterization and etiology of XX/XY blood chimerism in cattle, sheep and goats

Cell chimerism is defined as the presence of cell lines with different genetic backgrounds in the same animal. Occurring during fetal development, chimerism can result from embryonic implantation of maternal cells (or co-twin cells in multiple pregnancy), from the fusion of zygotes, or from simultaneous fertilization of both ovum and polar body by two spermatozoa (Ford, 1969; Jankowski and Ildstad, 1997). A special kind of cell chimerism is blood chimerism observed in animals from twin or multiple dizygotic pregnancies (independently of the gender of the fetuses) as a consequence of the exchange of hematopoietic tissue by fused of placental blood vessels (anastomoses). In the case of heterosexual twins with sex chromosome chimerism (XX/XY) such fusion facilitates migration of masculinization molecules (e.g. the SRY transcription factor, the Müllerian inhibiting substance – MIS, and

testosterone) produced by testicles to the female co-twin, which cause an abnormal development of the reproductive tract in the female fetus (Kozubska-Sobocińska et al., 2011; Szczerbal et al., 2014). In most cases, gonads develop into ovotestes that contain both ovarian and testicular tissues (defined as freemartinism, leading usually to female's infertility). The remainder of the internal reproductive tract is usually underdeveloped and can vary from an almost normal female to that very similar to a male (Kastli, 1974; Marcum, 1974; Long, 1990; Szatkowska and Świtoński, 1996; Szatkowska at al., 2004).

In cattle, the incidence of multiple pregnancies ranges from about 1% for beef breeds to approximately 5% for dairy breeds (Komisarek and Dorynek, 2002; Szczerbal et al., 2014), wherein, in the latter case, it is strongly affected by age and parity of the dam (from 1% for heifers to nearly 10% for older cows) (López-Gatius and Hunter, 2005; Des Coteaux et al., 2010). Double births rate can increase over a 10-year period for cows, and the largest increase is observed between the first and second parity (with a positive correlation between milk yield and multiple pregnancies) (Silva del Rio et al., 2007; Andreu-Vazquez et al., 2012). Selection for high milk yield favors multiplication of genes in a population that may also, to a certain extent, promote a predisposition to double ovulations, which increase the likelihood of twin pregnancy (resulting in more than 20% twinning in dairy cows) (Wiltbank et al., 2006; Bierman et al., 2010). The effect of twin pregnancy on the milk yield of cows is highly complex and depends on the nutritional requirements of pregnant cows, the levels of estrogenic hormones, progesterone, placental lactogen, prolactin, and the dry length period (Bicalho et al., 2007; Mostafa, 2009). One of the key aspects is that increasing milk production is associated with increased metabolism of the steroid hormones, which could be responsible for multiple ovulations (Wiltbank et al., 2006). In high efficiency lactating cows, high feed intake increases blood flow to the digestive tract and to the liver. Because blood entering the liver, the primary site of steroid metabolism, is cleared of the steroids, increased blood flow can influence the number of dominant follicles by affecting estradiol and, indirectly, FSH (follicle stimulating hormone) concentrations. Moreover, the twinning rate is slightly influenced by seasonal effects, believably affected by such factors as changes in temperature, duration of daylight, and feeding at conception (Sawa et al., 2012, 2015).

The first reports on XX/XY blood chimerism detected in leukocytes, classified as a separate karyotype abnormality in *Bovidae*, appeared in the literature in the early 1960s (Ohno et al., 1962; Fechheimer et al., 1963). The presence of two cell lines in the blood of cattle twins had been previously reported, and the diagnosed chimerism occurred in a population of erythrocytes (Stormont et al., 1958; Stone et al., 1960). In subsequent experiments, blood chimerism was diagnosed in the other farm animal species including sheep (Long, 1990; Kozubska-Sobocińska and Rejduch, 2008), goats (Szatkowska and Świtoński, 1996), pigs (Barasc et al., 2014), and horses (Demyda-Peyrás et al., 2013, 2014).

XX/XY cell chimerism and freemartinism syndrome

Freemartinism occurs in females born from twins or multiple pregnancies carrying fetuses of different gender, leading to disturbed development of the reproductive

system including infertility (Ford, 1969; Dunn et al., 1979; Long, 1990). The presence of several cell lines is due to the formation of a shared blood system through anastomoses or vascular connections between fetal membranes of co-twins before the sexual differentiation of the fetuses. As gonadal differentiation begins several days earlier in males than in females, it has been proposed that sex-determining factors (hormones) from the developing male gonad would be transported to the female fetus, potentially suppressing the reproductive organ development of the female. Such abnormalities of the female twin are caused by the AMH (anti-Müllerian hormone) secreted by fetal Sertoli cells. This hormone is probably responsible for the regression of the Müllerian structures in males as a part of their normal sexual development and can also be involved in the morphological differentiation of testes (Vigier et al., 1988). In heterosexual cattle twins, the regression of Müllerian ducts occurs simultaneously in males and freemartins between 50 and 80 days of pregnancy. The masculinization of the gonads and the development of the Wolffian ducts occur later, around 90 day (Komisarek and Dorynek, 2002). Both twins exhibit high serum AMH concentrations, whereas the gonadal production of this hormone in females is very low (Esteves et al., 2012). In addition, anastomoses allow for the interchange of hemopoetic stem cells between fetuses, resulting in the formation of blood chimerism in heterosexual twins (Padula, 2005).

Based on long-term cattle studies, it is assumed that 82–95% of heifers from heterosexual twin pregnancies have leukocyte chimerism and freemartinism (Marcum, 1974; Zhang et al., 1994). The remaining 5%–18% of females develop correctly, presumably because the placental anastomoses fail to fuse or because the fusion occurs following the critical period of reproductive organ differentiation (Szczerbal et al., 2014).

The sterility of females with a XX/XY karyotype is caused by extensive pathological changes in the reproductive system (Komisarek and Dorynek, 2002; Peretti et al., 2008; Villagómez et al., 2009). The external genitalia are, in most cases, typically female (Kastli, 1974; Long, 1990; Khan and Foley, 1994). The observed changes include an enlarged clitoris, a small, blind-ending vagina, hypoplasia, or aplasia of the uterus. Hypoplastic, sometimes masculinized gonads may contain ovotesticular structures (Marcum, 1974; Miyake et al., 1980; Zhang et al., 1994). However, the ratio of XX to XY karyotypes in freemartins varies considerably from individual to individual and is not related to the degree of masculinization (Nowacka et al., 2004; Szatkowska et al., 2004; Kozubska-Sobocińska et al., 2011; Szczerbal et al., 2014).

Numerous cases of freemartinism associated with leukocyte chimerism (54,XX/54,XY) were also noted in sheep (Szatkowska and Świtoński, 1996; Keszka et al., 2001; Kozubska-Sobocińska et al., 2003). As observed in cattle, female sheep exhibiting cell chimerism that originates from twin or multiple heterosexual pregnancies are infertile. The most common anatomical changes in ovine freemartins include enlarged clitoris, short, blind-ending vagina, the presence of testes and epididymis, and a uterus with different degrees of hypoplasia (Dain, 1971; Sysa et al., 1996).

The incidence of freemartinism in the sheep population is much lower than in cattle and ranges from 1.2% in Finnish Landrace to 11.2–25.14% in Booroola (Wilkes

et al., 1978; Keszka and Jaszczak, 1996). The background of placental anastomoses development is not clear, however there are indications that breed type may influence the incidence of chimerism in cattle (Komisarek and Dorynek, 2002). There are also reports suggesting hereditary tendency to develop placental anastomoses between co-twins in sheep, but it should be pointed out that these reports are based on single cases, found in inbred herds (Dain, 1971; Szatkowska and Świtoński, 1996; Keszka et al., 2001).

In goats freemartinism is even less frequently associated to a 60,XX/60,XY karyotype than in sheep (about 1% of different-sex twins) (Yadav et al., 1993). Female goats having XX/XY chimerism were characterized by enlarged clitoris, rudimentary vagina, hypoplastic uterus, and the presence of scrotal testes (Omura, 1977; Bondurant et al., 1980; Padula, 2005). Postmortem examination of one of these freemartins showed the presence of pseudovagina, seminiferous tubules, and deferent ducts. No residual Müllerian ducts were observed (Smith and Dunn, 1981).

XX/XY chimerism and male fertility

The presence of female co-twins during embryogenesis on the subsequent reproductive performance of males from twins or multiple different-sex pregnancies has been the subject of much debate and analysis. Numerous studies with bovine twins demonstrated that 60,XX/60,XY leukocyte chimerism generally does not affect bull fertility and thereby males can be conditionally accepted for breeding (Gustavsson, 1977; Jaszczak et al., 1988; Kovács and Karakas, 1997). Those studies showed that the semen from chimeric bulls did not differ in quality or suitability for freezing compared to that from animals with normal karyotype. However, conflicting reports indicated reduced fertility parameters in bulls with 60,XX/60,XY chimerism (Dunn et al., 1979; Świtoński et al., 1991). In particular, a study by Cribiu and Popescu (1982) showed that the non-return rate in chimeric bulls was significantly decreased in 50% of chimeric animals compared with bulls with a normal karyotype. Other authors also observed that fertility parameters are considerably decreased in chimeric bulls. Additionally, when evaluating semen quality, Dunn et al. (1979) reported that sperm motility and concentration were considerably lower in ejaculates collected from bulls with 60,XX/60,XY karyotype, as well as higher incidence of degenerative changes in testicular structure, when compared with control animals.

The analysis of semen parameters such as ejaculate volume, sperm motility and concentration, and percentage of damaged sperm (primary and secondary morphological defects) performed by Rejduch et al. (2011) also showed these parameters were lower in bulls with 60,XX/60,XY chimerism compared to bulls with 60,XY karyotype that were kept under the same conditions. More specifically, this comparison showed statistically significant differences with regard to the volume of ejaculate and statistically highly significant differences concerning the motility of spermatozoa and sperm concentration (Table 1) (Rejduch et al., 2011).

The results of Dunn et al. (1979) were confirmed by Świtoński et al. (1991), that showed that a group of chimeric bulls presented poorer reproductive performance, including a decrease in non-return rate and in the percentage of ejaculates accepted for freezing as well as a higher number of discarded semen samples after freezing/

thawing. Nevertheless, the authors suggested these sires could be, in justified cases, conditionally qualified for use in A.I. stations.

Table 1. Comparison of semen parameters of bulls with leukocyte chimerism 60,XX/60,XY and bulls with karyotype 60,XY (Rejduch et al., 2011)

Group	XY cell line in leukocytes (%)	Spermatozoa with morphological defects (mean %)		Mean volume (ml)	Mean motile spermatozoa (%)	Mean sperm concentration/ml ($\times 10^6$)
		major	minor			
Experimental (60,XX/60,XY) n=15	8–97	8.17	17.88	3.72*	54.85**	762**
Control (60,XY) n=15	100	5.30	6.40	4.39	70.1	1113

* significant differences ($P<0.05$).
** highly significant differences ($P<0.01$).

It has been hypothesized that early dissociation of the XY bivalent may reduce male fertility (Świtoński and Stranzinger, 1998; Villagomez and Pinton, 2008; Villagómez et al., 2009), therefore bulls and rams with a normal karyotype and reproductive problems were compared with males with 60,XX/60,XY and 54,XX/54,XY karyotypes, respectively (Rejduch et al., 2000 a, b), and cytomolecular analysis (FISH) of meiotic chromosome pairing in gonads was performed (Rejduch et al., 2000 a; Rejduch and Kozubska-Sobocińska, 2006). According to Rejduch et al. (2000 b), early dissociation (at prophase or metaphase of the first meiotic division) of the sex bivalent occurs in 2.0 to 5.6% of spermatocytes in bulls with XX/XY chimerism and in 3.1% of spermatocytes in bulls with 60,XY karyotype. In rams with 54,XX/54,XY karyotype, the early dissociation of the XY bivalent ranged from 11.1% to 19.0%, compared to 3.4% in rams with a normal karyotype (Rejduch et al., 2000 b). Although the frequency of early dissociation of the sex bivalent in males with normal karyotype reported by other authors was similar in bulls (3.6%; Świtoński et al., 1991) it was lower in rams (3.4%; Dai et al., 1994). However, the latter quoted result was considerably higher than the values of 0.5%–2.5% observed in normal rams by Kozubska-Sobocińska et al. (2009). It should be noted that in males with normal karyotype, early dissociation of the XY bivalent is a rather rare occurrence, likewise in the case of carriers of leukocyte chimerism, but it concerns only a small number of animals tested so far. Therefore, in light of these results, the effects on male fertility of bulls born in heterosexual twinning are still discussed.

Evidences are inconclusive with respect to the effect of 54,XX/54,XY chimerism on ram fertility. Rynkiewicz-Szatkowska (1992) have described two rams with leukocyte chimerism that showed poor semen parameters, including the ejaculate volume, sperm motility and concentration and percentage of spermatozoa with primary and secondary morphological defects. However, no reliable information confirming these observations exists in the current literature.

Limited information is available on the effects of 60,XX/60,XY chimerism over fertility traits in bucks, possibly due to the low frequency of freemartinism occurrence in this species (Szatkowska et al., 2004). Furthermore, it is worth noting that there are some cases reported of true or whole body chimerism in infertile goats (Pailhoux et al., 1994; Batista et al., 2000).

Diagnosis of cell chimerism

In recent years, the diagnosis of cell chimerism has been focused mainly on cytogenetic and molecular analyses. Multiple attempts have been made determining the usefulness of each of these approaches for identifying different cell lines in blood and other tissues.

Cytogenetic diagnosis of chimerism is based on cell lines karyotyping and identification of heterosomes using classical staining methods of metaphase or prometaphase chromosomes, both conventional and G/Q or R and C banding (Iannuzzi and Di Meo, 1995; Esteves et al., 2012). Such diagnosis, requiring analysis of at least several hundred metaphases is a laborious and difficult procedure, taking into account a large diploid chromosome number and their complex morphology (Iannuzzi and Di Berardino, 2008; Peretti et al., 2008). Moreover, the traditional methods are often inaccurate in identifying sex chromosomes, therefore their precise and unambiguous determination in mitotic and meiotic cells requires the application of fluorescent *in situ* hybridization technique (FISH) (Rubeš et al., 2009; Villagomez and Pinton, 2008) with X- and Y-specific molecular probes (Kobayashi et al., 1998; Słota et al., 2003; Di Berardino et al., 2004; Kozubska-Sobocińska et al., 2012). This technique, specially applied in interphase cells (Sohn et al., 2007; Vorsanowa et al., 2010), provides a fast and strict pinpointing of sex chromosomes, which are the markers of XX and XY cell lines in chimeric animals from different gender twin pregnancies (Demyda-Peyrás et al., 2013, 2014) as well as enables estimation of the proportion of the co-twin line (Demyda-Peyrás et al., 2013, 2014). However, commercial FISH heterosome probes are available only for a few livestock species and the high costs prevent their use for routine cytogenetic analyses.

On the other hand, a fast, sensitive, less time and cost consuming PCR-based molecular analysis (mini- and microsatellite DNA polymorphisms) can be considered as a powerful tool in the successful detection of chimerism (Grobert et al., 1992; Plante et al., 1992; Rejduch et al., 2004), especially the use of routinely practiced parentage short tandem repeat (STR) tests (multiplex PCR with the available commercial sets of STR markers distributed along the whole genome) (Demyda-Peyrás et al., 2013). In that case it is recommended to analyze DNA isolated from blood cells and hair follicles with the aim to recognize one's own and full-sib cell lines. However, this approach has some limitations because it is insensitive to distinguish between chimeras caused by the cell exchange between hetero- and homosexual twins. Additionally, the analysis of X- and Y-linked gene (*SRY, AMELX/AMELY, ZFX/ZFY*) polymorphisms (Mc Niel et al., 2006; Martinez-Royo et al., 2009; Pourjafar et al., 2012; Demyda-Peyrás et al., 2014) or anonymous markers assigned to the Y heterosome (Kakoi et al., 2005; Ron et al., 2011) may be also used to detect Y chromosome in XX/XY females. Nevertheless, if the Y-linked markers are only studied in blood

cells it does not facilitate to distinguish between the XX/XY chimerism and other disorders of sex development (DSD), i.e. sex reversal syndrome (female with a male karyotype) (Szczerbal et al., 2014).

In general, the analysis of the Y-linked marker polymorphism along with STR markers localized on autosomes both in blood and in hair facilitates the detection of chimerism. The highest informativity and good accuracy for chimerism assessment is offered by the use of parentage QF-PCR based STR screening (quantitative fluorescence PCR) (Vodicka et al., 2004; Donaghue et al., 2010; Xu et al., 2013) or more sensitive Real-Time PCR assay (real-time quantitative reverse-transcription PCR) (Alizadeh et al., 2002), supplemented by STR linked to the sex chromosomes. According to these methods, different allele patterns in QF-PCR STR analysis in blood and hair samples can be explained by coexistence of two different cell lines in the same animal. Therefore, QF-PCR technique could be suggested as a definitive diagnostic tool for determining cellular chimerism in several livestock species (Martinez-Royo et al., 2009; Demyda-Peyrás et al., 2013). However, to avoid ambiguities in cases of freemartins and other DSD, particularly in single born females with underdevelopment of internal reproductive tracts (Szczerbal et al., 2014), it is suggested the diagnosis should be performed with the use both cytogenetic and molecular methods as a combined strategy that allows early, rapid detection of sex chromosome chimerism, saving time, efforts and financial outlays (Nowacka et al., 2004; Demyda-Peyrás et al., 2013, 2014).

Another, the most recent molecular method which can be considered as a valuable tool of identification of chimerism is genome-wide single nucleotide polymorphism (SNP) array analysis in various biological materials (Craig et al., 2009). The SNP array uses a combination of intensity (genomic dosage) and genotyping data from different tissues that provide high-resolution means to the differentiation of chimerism and mosaicism, as the additional presence of extra genotypes in the chimeras is readily detectable. Concretely, in the case of blood chimerism (associated with the occurrence of anastomoses) the mixed genetic profiles present in one blood sample can be easily recognized by the analysis of intensity parameters (copy number), which deviate from the expected values (Gurgul et al., 2014). The straightforward SNP micro-array analysis of unstimulated blood offers several advantages for detection of chimerism (higher sensitivity) compared with cytogenetic or CGH (comparative genomic hybridization) assay (Holl et al., 2013) in which a lot of cells can be surveyed at once, since DNA is extracted from a culture of many (interphase and metaphase) cells, eliminating the culture bias introduced by evaluation of metaphase cells only (Conlin et al., 2010).

The diagnosis of blood chimerism has been earlier analyzed using immunological methods which practically have been recently replaced by molecular approaches, giving unambiguous results (Esteves et al., 2012). The fundamental immunological diagnosis is based on erythrocyte antigens but this test often yields inconclusive results because of non-specific hemolysis in co-twins (Kastli, 1974). The other immunological method concerns the H-Y antigen (occurring in plasma membranes of male haploid germ cells) and involves a cytotoxicity test, in which the female T-lymphocytes are sensitized to this antigen (Wachtel et al., 1980). In females from

different sex twin or multiple pregnancies, the positive test (H-Y$^+$) is usually detected, meaning the male-specific H-Y antigen is present (Utsumi and Iritani, 1993).

In turn, the endocrinological diagnosis of chimerism in females from dizygotic twins or triplets concentrated on the use of sex steroids (derivatives of testosterone) in the peripheral blood to determine the endocrine sex. The presence of AMH (anti-Müllerian hormone) is also of importance, as its expression during embryogenesis inhibits the formation of Müllerian ducts, and is associated with male gonad development (Rota et al., 2002). Anastomoses that form between fetal membranes of ruminant pluriparous pregnancies provide an anatomical basis not only for exchange of hematopoietic tissue cells but also for hormonal impact and the effect of AMH on co-developing embryos (Padula, 2005).

The hormonal methods mentioned above may be to a small extent useful for establishing a diagnosis of freemartinism, confirming endocrinological functionality of gonadal tissue, and predicting potential future fertility. However, considering the varying degrees of masculinization associated with freemartin syndrome these tests lack specificity (Esteves et al., 2012), and therefore nowadays they are only occasionally used for detection of freemartinism (Pourjafar et al., 2012).

Cytogenetic and molecular diagnostics of XX/XY chimerism in *Bovidae*

Breeding animals are submitted to cytogenetic studies identifying chromosomal aberrations and karyotype defects, which in general have an adverse effect on animal fertility and development (Danielak-Czech and Słota, 2004, 2008; Villagomez and Pinton, 2008; Villagomez et al., 2009). Leukocyte chimerism is a specific type of karyotype abnormality with the formula 2n,XX/2n,XY, which is diagnosed by identifying cell lines differing in sex chromosomes (Iannuzzi and Di Berardino, 2008; Peretti et al., 2008).

This abnormality can be easily diagnosed in cattle karyotype due to the specific heterosome morphology (Figure 1) (Słota et al., 2000; Iannuzzi and Di Berardino, 2008), but the identification of an acrocentric X chromosome in sheep and goats or an acrocentric Y heterosome with small p-arms in zebu requires the use of differential staining techniques such as CBG, GTG, RBA and QFQ (Iannuzzi and Di Meo, 1995; Słota et al., 2001; Biswas et al., 2015). The CBG technique distinguishes the X heterosome from autosomal chromosomes due to its specific feature of a very pale C band in the centromeric heterochromatin region (Kozubska-Sobocińska et al., 2003).

The classic cytogenetic methods represent a specific test for freemartin syndrome that can be used in young animals suspected to be a freemartin upon a clinical examination or with history of being born co-twin to a male. Nevertheless, it is worth noting that in cases where XX/XY chimerism has occurred with relatively low frequency of XY, it is important to examine many hundreds of metaphase spreads to be certain of the freemartinism diagnosis (Markum et al., 1974; Zhang et al., 1994; Perretti et al., 2008; Demyda-Peyrás et al., 2013). In this context, it appears that classical cytogenetic approach is really expensive since it is quite time demanding and the laboratory personnel need to be quite qualified (especially in diagnostics of sheep and goats). However, these standard techniques allow for the discovery of other chromosomal abnormalities (such as balanced chromosome mutations or sex

chromosome aneuploidies), which occasionally coincide with XX/XY chimeras in blood tissue (Słota et al., 2004; Esteves et al., 2012; Demyda-Peyrás et al., 2014; Danielak-Czech et al., 2016).

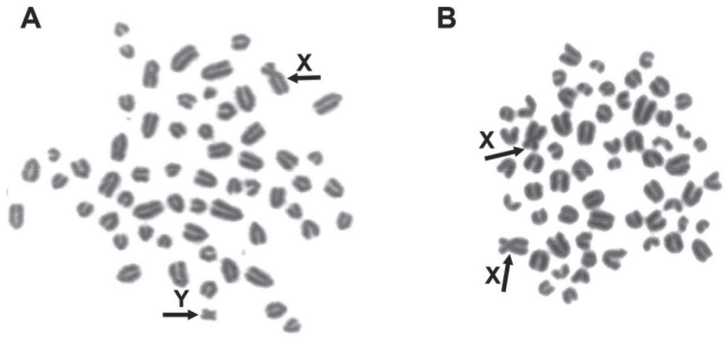

Figure 1. Metaphase chromosomes of a bull with 60,XX/60,XY karyotype: cell lines 60,XY (A) and 60,XX (B) (A. Kozubska-Sobocińska)

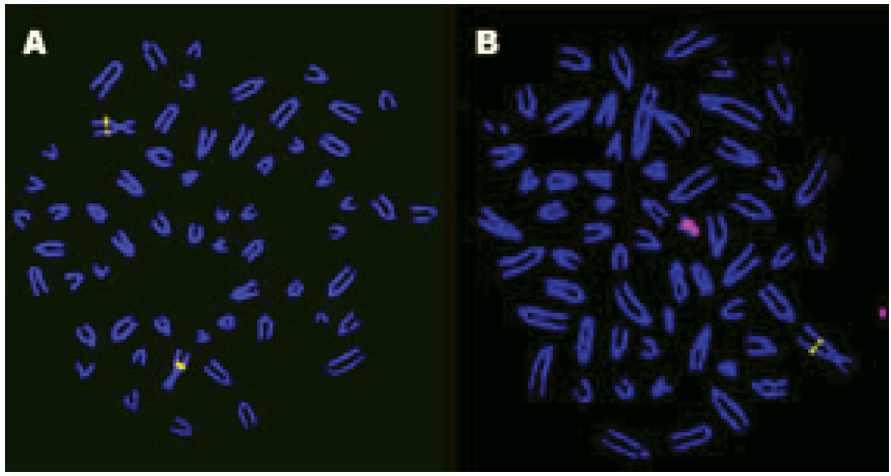

Figure 2. Metaphase chromosomes of a bull with 60,XX/60,XY karyotype. FISH technique with X-biotin/avidin-FITC and Y-digoxigenin (DIG) labeled probes: yellow (FITC) fluorescence signals on X chromosomes in 60,XX cell line (A) and yellow (FITC) and red (DIG) fluorescence signals on X and Y heterosomes in 60,XY cell line (B) (Kozubska-Sobocińska et al., 2011)

Today, molecular chromosome studies using FISH are becoming increasingly applied in cytogenetic diagnosis (Figure 2). This method can be used for analysis of small structural mutations and chromosomal region polymorphisms (Rubeš et al., 2009), physical gene mapping (Danielak-Czech et al., 2014 a, b), and identification of sex chromosomes in somatic cells (mainly leukocytes) and germ cells (in gonads or sperm) (Hassanane et al., 1999; Revay et al., 2002), which are markers of female and male cell lines (Di Meo et al., 2005; Rejduch and Kozubska-Sobocińska, 2006).

Fluorescence *in situ* hybridization technique is also increasingly used for semen analysis because it is the most reliable method to identify heterosomes in gametes, making it possible to determine the percentage of aneuploid sperm in the ejaculate (Nicodemo et al., 2009; Pauciullo et al., 2011). It also allows for the observation of the segregation of chromosomes to daughter cells (Figure 3) (Rejduch et al., 2005).

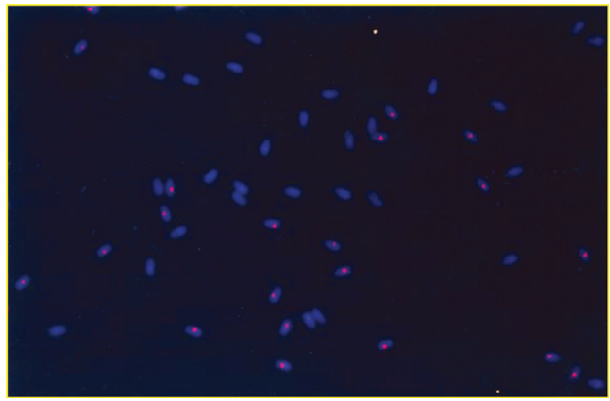

Figure 3. Sperm-FISH with Y-digoxigenin (DIG) labeled probe; fluorescent red-purple signals in the semen of ram identify Y chromosomes (Rejduch et al., 2005)

The application of FISH enabled the identification of heterosomes in semen samples of three Romanov rams from multiple heterosexual pregnancies, in which 54,XX (21%–35%) / 54,XY (65%–79%) leukocyte chimerism was identified based on the analysis of metaphase chromosomes (Kozubska-Sobocińska and Rejduch, 2008). The hybridization signal analysis performed in 1000 spermatozoa from each ram, revealed the proportions of spermatozoa containing the X (47%–52%) or Y (43%–46%) heterosome in the haploid set, as well as the percentage (5%-9%) of aneuploid spermatozoa, displaying no signal. Similarly, this technique also showed the existence of similar proportions between spermatozoa with X and Y heterosomes in bulls with 60,XX/60,XY karyotype (ranging from 45% : 55% to 57% : 43%, respectively) (Rejduch et al., 2011). FISH-based determination of the X- and Y-bearing sperm fractions in the semen of bulls and rams with XX/XY chimerism at a level of approximately 50% (Kozubska-Sobocińska and Rejduch, 2008; Rejduch et al., 2011) denied the hypotheses of Lojda (1972) and De Giovanni et al. (1975) who suggested that females were more numerous (75–78%) among the offspring of leukocyte chimerism carriers in *Bovidae*.

The presence of the XY line in females could be obtained from different-sex twins searching for DNA fragments specific to the Y chromosome (e.g. *SRY*, *AMELY,* and *ZFY* genes or microsatellite sequences) in PCR technique (Utsumi and Iritani, 1993; Pailhoux et al., 1994; Szczerbal et al., 2014), as well as heterosome-specific molecular probes in FISH method (Rejduch et al., 2005; Sohn et al., 2007).

For diagnosing cell chimerism in sheep and goats, additional tools are provided by genetic conservatism which, in *Bovidae*, consists of erythrocyte antigens (Rychlik et al., 2005), STR (Edwards et al., 2000; Kozubska-Sobocińska et al., 2008), and cytogenetic markers (Di Berardino et al., 2004; Pauciullo et al., 2011). Interspecific *in situ* hybridization with bovine probes was used for identification of the ratio of XX and XY leukocyte cell lines in goat chimeras (63% : 37%) (Rychlik et al., 2005). This technique was also used for mark X and Y chromosomes in river buffalo, sheep, and goat spermatozoa (Di Berardino et al., 2004).

A comprehensive diagnosis of cell chimerism performed by Rejduch et al. (2004) in Romanov sheep included lambs from multiple pregnancies (84 twin pairs, 3 triplet litters, and quadruplets). The analysis, carried out using three different methods, showed cell chimerism in four pairs of twins (4.8%), in all animals of one triplet, and in two animals of the quadruplet. Based on the analysis of erythrocyte antigens from six blood group systems, nine cases of erythrocyte chimerism were found (in two pairs of same-sex twins, in different-sex triplets, and in a ram and ewe from quadruplets) (Rejduch et al., 2004). On the other hand, the polymorphism analysis of six microsatellite *loci* (*BMS360*, *INRA123*, *McM42*, *CSSM66*, *ETH225,* and *TGLA53*) revealed cell chimerism in 13 lambs: three pairs of same-sex twins, a pair of different sex twins, different-sex triplets, and a ewe and ram from quadruplets (Rejduch et al., 2004). In turn, in the same group of lambs FISH enabled seven cases of leukocyte chimerism to be diagnosed in all animals from multiple different pregnancies (a pair of different-sex twins, different-sex triplets, and a ram and ewe from a quadruplet litter), which had been previously diagnosed with cell chimerism based on genotypes at DNA microsatellite *loci* (Rejduch et al., 2004).

In a study of blood chimerism in goats, Rychlik et al. (2005) used genetic conservatism involving erythrocyte antigens. From a set of test reagents used for blood typing in sheep, seven reagents (anti-Aa, -Be, -Bi, - Bd, -Bb, -Ca, -R) were chosen to diagnose erythrocyte chimerism in goat kids from multiple pregnancies. Fifteen pairs of homozygotic or dizygotic twins were investigated, and based on incomplete reactions in the hemolytic test, two pairs of chimeric (same-sex and different-sex) twins were identified. This method of chimerism diagnosis can be replaced by more reliable molecular technique based on multiplex PCR assay of STR markers polymorphism for parentage control supplemented with X- and Y-specific markers (Demyda-Peyrás et al., 2014), but in goats this approach has not been routinely applied until now.

Conclusions

The current diagnostic techniques for identification of blood chimerism most heavily rely on cytomolecular methods (cell culture, chromosome banding, FISH), which are labor-intensive and have a significant failure rate, especially when sample quality is poor.

The incorporation of modern molecular and cytogenetic techniques for cell chimerism studies provides an increase in the diagnostic potential of determining the consequences of XX/XY chimerism in cattle, sheep, and goats.

The molecular automated techniques, such as multiplex PCR and QF-PCR STR polymorphism or straightforward SNP micro-array are more efficient and cost-effective testing strategies with a higher diagnostic success rate than karyotype evaluation.

The precise diagnostics of blood chimerism may provide a basis for formulating accurate guidelines and selection recommendations concerning the reproductive performance of sires with XX/XY karyotype.

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