Identification of heterosomes in spermatoza of rams with 54,XX/54,XY chimerism

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ABSTRACT: The aim of the study was to identify heterosomes in the semen of three Romanov rams – carriers of leukocyte chimerism (FISH technique) and to determine the proportions between spermatozoa with X and Y chromosomes. The choice of bovine probes for hybridization with ram heterosomes was dictated by genetic conservatism of bovine and ovine heterosomes. The ratio between spermatozoa with a yellow fluorescent signal containing the X chromosome in the haploid set and spermatozoa with a red-purple signal indicating the presence of the Y chromosome, taking into account spermatozoa with no signal, was 52%:43%:5% in ram No. PL100006077676; 47%:44%:9% in ram No. PL100006078031; and 48%:46%:6% in ram No. PL100006078895. The results obtained lead us to conclude that the 54,XX/54,XY chimerism has no effect on sex ratio in offspring.

Keywords: sheep; leukocyte chimerism; FISH; spermatozoa; heterosomes

XX/XY leukocyte chimerism is the most common karyotype defect in Bovidae. It involves the presence of two cell populations with a male and female karyotype in the blood of animals from multiple different-sex pregnancies, and results from a common blood circulatory system being formed through anastomoses between fetal membranes of co-twins (Rejduch, 2001).

In animals, cell chimerism is diagnosed based on erythrocyte antigens, microsatellite DNA sequences and sex hormone levels. Most often, however, leukocyte chimerism is diagnosed cytogenetically using heterosome analysis in metaphase plates and identification of two cell lines differing in sex chromosomes (Rejduch, 2001; Rychlik et al., 2005).

Recently, fluorescent *in situ* hybridization (FISH) has been increasingly applied to evaluate chromosomes, to diagnose structural mutations, to evaluate the polymorphism and genetic conservatism of chromosome regions, and to identify chromosomes obtained from somatic and sex cells (Mohaddes et al., 1996; Rejduch et al., 2000; Rejduch, 2001; Di Berardino et al., 2004; Kozubska-Sobocinska et al., 2005; Rychlik et al., 2005).

The FISH technique is also increasingly used to evaluate the semen of males with chromosome changes detected during karyotype analysis, because it enables the percentage of aneuploid spermatozoa to be determined in ejaculates (Christensen and Bruusgaard, 1999; Di Berardino et al., 2004; Vidal et al., 2004).

The aim of the study was to perform cytogenetic analysis of sex chromosomes in the semen of rams with leukocyte chimerism using FISH technique and to find out if and how 54,XX/54,XY chimerism affects the sex ratio in offspring.

MATERIAL AND METHODS

The study involved ejaculate samples taken in the autumn season (and stored at –20°C) from three Romanov rams (PL100006077676, PL100006078031 and PL100006078895) born from multiple differ-

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ent-sex pregnancies, which during FISH analysis of metaphase chromosomes were found to carry 54,XX/54,XY leukocyte chimerism (Figure 1).

To thaw semen samples, 9 ml of 0.9% NaCl was added to every tube and mixed softly for 3 min. Thawed samples were centrifuged for 5 min. (3 000 rev/min) and 0.5 ml of 0.9% NaCl was added after removal of supernatant. The suspension obtained was thoroughly mixed using a pipette.

Preparations of spermatozoa were obtained according to protocol described by Blotter et al. (1990).

One drop of ejaculate was placed on a defatted glass slide and spread all over the second slide. Preparations were dried vertically at room temperature.

Preparations were immersed for 12–17 h in 95–99.5% ethyl alcohol and dried vertically.

Sperm was decondensed in a dithiothreitol (DTT) and papainase solution for 4–8 min, and the preparations were immersed twice in 0.2M Tris solution. After decondensation, the preparations were rinsed three times in 95–99.5% ethyl alcohol and dried vertically.

The degree of sperm decondensation was analysed under a contrast phase microscope. Where sperm image was clear, the preparations were fixed using a methanol and acetic acid mixture (3:1) for 12–17 h.

To identify the heterosomes in ram spermatozoa, FISH was applied following the procedure of Pinkel et al. (1986) and Solinas-Toldo et al. (1993), using bovine X and Y specific probes. These probes were obtained through microdissection of 10 heterosomes X and Y (with average degree of spiralization) from metaphase plate preparations obtained from bovine lymphocyte culture. The X probe was amplified and labelled with DOP-PCR using the bio-16-dUTP nucleotide (Goldammer et al., 1996). The Y probe was amplified by PCR as described by Guan et al. (1992) and PCR labelled with digoxigenin-11-dUTP (Boehringer Mannheim, Germany) according to the manufacturer's instruction.

Prior to hybridization, the slides with spermatozoa were treated with RNAse (1 mg/ml) at 37°C for 60 min, sequentially washed in 2 × SSC buffer (0.3M NaCl and 0.03M sodium citrate, pH 7.0) and dehydrated in 70%, 80%, 90%, and absolute ethanol for 2 min each. After that the cells were denatured by immersion in 70% formamid/2 × SSC at 70°C for 2 min, and then dehydrated by passing through a cold ethanol series. Just before hybridization, the Y-probe (60 ng), the X-probe (100 ng), sonicated bovine DNA (100 ng) and sonicated salmon sperm DNA (100 ng) were pooled together, dried down to 4.5 µl and mixed with 10.5 µl preincubated MMI (50% formamide, 10% dextran sulfate/1 × SSC, pH 7.0). The hybridization mixture was denatured at 75°C for 10 min in dry block and immediately chilled on ice for 5 min. Four microliters were applied on each slide under a 18 × 18 mm coverslip, mounted with rubber cement and kept overnight in a moist, dark chamber at 37°C. As a control of the hybridization experiments, we hybridized

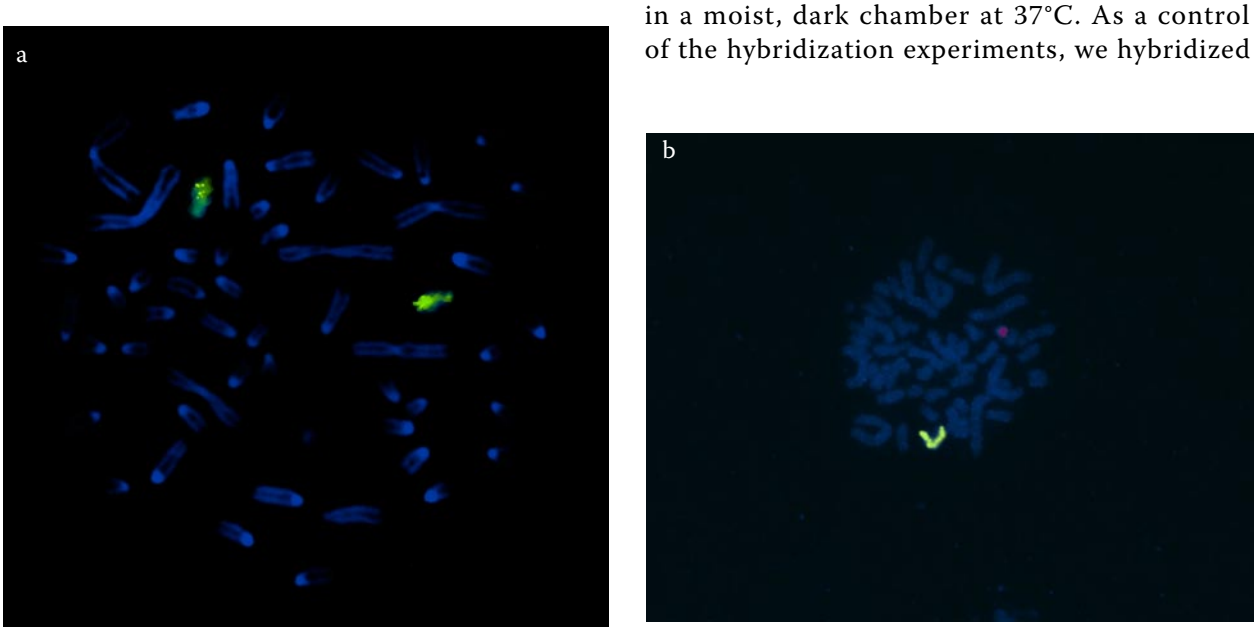

Figure 1. Signals identifying heterosomes on metaphase plates of ram No. PL100006077676: a – two yellow signals in 54,XX cell line; b – yellow and red-purple signals in 54,XY cell line

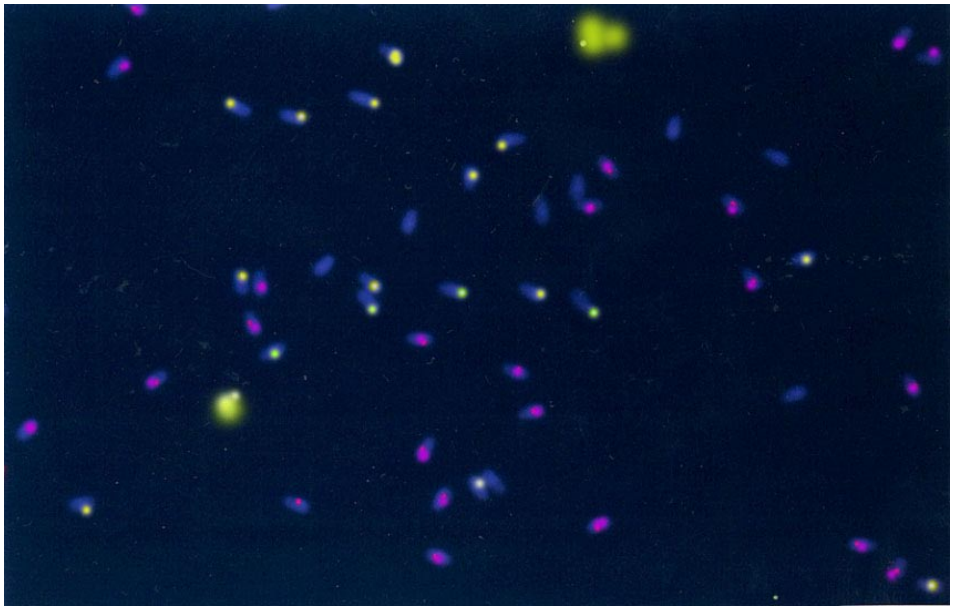

Figure 2. FISH technique. Fluorescent signals in the semen of ram No. PL100006077676: yellow signals identify X chromosomes, red-purple signals identify Y chromosomes

in parallel, somatic metaphases for comparison. Posthybridization washing was carried out for 8 min each in 59% formamide/2 × SSC, with a pH of 7.0 at 43–45°C.

The preparations were stained with 10 µl of antifade solution containing 20 µl of DAPI (4,6-diamidyno-2-phenylindol)/1 ml and observed under a fluorescent microscope (OPTON – Axiophot) using triple attenuation filters (DAPI/FITC/Texas Red). Selected cells were recorded and evaluated using the image analysis system LUCIA – FISH (Laboratory Imaging Ltd, Prague, Czech Republic).

RESULTS AND DISCUSSION

The hybridization of bovine heterosome painting probes with genetic material found in ram semen revealed clear single fluorescent signals: yellow on sperm with X chromosome, and red-purple in cells with Y heterosome (Figure 2).

Based on analysis of hybridization signals evaluated in 1 000 spermatozoa of every animal and using the image analysis system LUCIA – FISH, the proportion of sperm with a yellow fluorescent signal that identified the X chromosome in the haploid set, and the proportion of sperm with a red-purple signal indicating the presence of the Y chromosome were determined, taking into account those spermatozoa for which no signals were recorded (Table 1).

The FISH technique is the most reliable method of heterosome identification in male gonadal cells, as shown by an experiment using fluorescent *in situ* hybridization for analysis of chromosomes in spermatogonial cells in bulls with 60,XX/60,XY leukocyte chimerism (Rejduch et al., 2000). Hybridization with a double-labelled molecular probe (for X with biotin and for Y with digoxygenin) in six spermatogonial cells obtained from the testes of two bulls revealed no signals identifying Y chromosomes, and double signals corresponding to the X chromo-

Table 1. Results of analysis of fluorescent signals in the rams spermatozoa

No. of ram	Percentage of 54,XX/54,XY line in leukocyte cells	Number of spermatozoa analysed	Number of cells with X identifying signal (%)	Number of cells with Y identifying signal (%)	Number of cells without signal (%)
PL100006077676	21; 79	1 000	520 (52%)	429 (43%)	51 (5%)
PL100006078031	35; 65	1 000	472 (47%)	440 (44%)	88 (9%)
PL100006078895	28; 72	1 000	478 (48%)	463 (46%)	59 (6%)

somes were observed. The morphology of DAPI-stained chromosomes, on which the signals were located, also corresponded to X heterosomes in cattle. These results were confirmed in 2001 by studies involving a larger population of bulls born from twin different-sex pregnancies (Rejduch, 2001), resulting in the identification in five animals of signals indicating two X chromosomes on spermatogonial cells and the determination of a 4–11% proportion of a line containing XX heterosomes in relation to XY in the spermatogonia of the bulls analysed.

At present, the FISH technique is also used for semen analyses as it enables the proportion of spermatozoa containing the X or Y chromosome in the haploid set to be determined. Simultaneous hybridization performed in the present study with variously coloured probes identifying heterosomes in the semen of rams with leukocyte chimerism made it possible to accurately determine (based on analysis of 1 000 cells in every animal) the percentage of sperm with X or Y chromosome as well as the percentage of cells with no signal.

Simultaneous hybridization using two probes (labelled with biotin for X chromosome and digoxygenin for Y chromosome), performed in bull spermatozoa by Hassanane et al. (1999) made it possible to determine approx. 0.1% of disomic spermatozoa (0.067% XX, 0.029% YY and 0.029% XY) in the analysed sperm in addition to determining the X:Y ratio.

The choice of bovine probes for hybridization with ovine heterosomes was not accidental. It was motivated by genetic conservatism, which enables the use of several molecular probes obtained from one species to map single genes or whole chromosome fragments in taxonomically distant species (Revay et al., 2002; Di Berardino et al., 2004; Rejduch et al., 2004; Kozubska-Sobocinska et al., 2005). The possibility of using molecular probes from different species of animals significantly reduces the costs of analysis, which is an important aspect of practical studies.

The high conservatism of heterosomes in Bovidae was shown by Revay et al. (2002), who used the FISH technique to identify sex chromosomes in spermatozoa of 15 bulls using probes obtained by sorting heterosomes of the yak (*Bos grunniens*). Likewise, Di Berardino et al. (2004), who used interspecific hybridization, showed the usefulness of bovine probes specific to heterosomes (X- and Y-chromosome painting probe) for sexing and analysis of the semen of species such as river buffalo (*Bubalus bubalis* L.), sheep (*Ovis aries* L.) and goat (*Capra hircus* L.). Studying three animals of each species and 1 000 sperm per animal, Di Berardino et al. (2004) showed that the efficiency of the FISH technique exceeds 99% (Di Berardino et al., 2004). A similar experimental group was formed by three rams with 54,XX/54,XY karyotype, analysed in the present study. This is considered a representative sample considering the incidence of leukocyte chimerism in several to more than ten percent of animals (depending on breed) born from multiple different-sex pregnancies (Keszka et al., 2001).

The use of the FISH technique for identification of heterosomes in the semen of rams diagnosed with 54,XX/54,XY leukocyte chimerism provides new insights for the debate on uneven sex distribution among the offspring of males with chimerism. An evident superiority of females (75–78%) over males among the offspring of 60,XX/60,XY bulls, reported by Lojda (1972) and DeGiovanni et al. (1975), was considered as an indirect proof that reproductive cells move between twins. However, these suggestions were not supported by Fejer and Kovacs (1980) and by the analysis of secondary spermatocytes in bulls with chimerism, which showed that the X to Y heterosome ratio in the analysed metaphases was 1:1 (Rejduch, 2001). This shows that the presence of 54,XX/54,XY chimerism in rams does not change the proportion of spermatozoa with X or Y chromosome.

The results obtained in the present study correspond with the results of hybridization performed by Rejduch et al. (2005), who used only one probe identifying the Y chromosome in ram spermatozoa. Assuming that the cells in which no signal was observed contained the X heterosome in the haploid set, the ratio of X chromosome cells (55.5%) to Y chromosome cells (45.5%) was similar to that obtained in the present study. The present findings also allow a conclusion that 54,XX/54,XY chimerism has no effect on sex distribution in the offspring and all the deviations are accidental.

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