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Original article

Specific cytogenetic labeling of bovine spermatozoa bearing X or Y chromosomes using fluorescent *in situ* hybridization (FISH)

François PIUMI[a,*], Daniel VAIMAN[b], Edmond-Paul CRIBIU[b], Bernard GUÉRIN[a], Patrice HUMBLOT[a]

[a] UNCEIA, Services techniques, Maisons-Alfort, France
[b] Département de génétique animale, Laboratoire de génétique biochimique et de cytogénétique, Groupe petits ruminants, Institut national de la recherche agronomique, 78352 Jouy-en-Josas, France

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Abstract – X and Y specific probes were identified in order to apply the fluorescent *in situ* hybridization (FISH) technique to bovine spermatozoa. For Y chromosome detection, the BRY4a repetitive probe, covering three quarters of the chromosome, was used. For X chromosome detection, a goat Bacterial Artificial Chromosome (BAC) specific to the X chromosome of bovine and goats and giving a strong FISH signal was used. Each probe labeled roughly 45% of sperm cells. The hybridization method will be useful for evaluating the ratio of X- and Y- bearing spermatozoa in a sperm sample and consequently can be used to evaluate the efficiency of sperm sorting by different techniques such as flow cytometry.

fluorescent *in situ* hybridization / spermatozoon / X chromosome / Y chromosome / bacterial artificial chromosome

1. INTRODUCTION

In mammals, sex is determined at fertilization by the genomic content of the spermatozoa. Manipulations of the sex-ratio are based either on sex determination on embryonic material [14] or on sperm cell sorting [7]. In livestock, there is a clear interest in producing animals of one sex or another. This is particularly true in cattle, where male progeny is preferable for beef breeding or to produce selected sires in selection schemes, whereas females are desired in dairy production. Similarly, gilts are more efficient than castrated pigs for food conversion in swine. The only exploitable difference in sperm cells is the DNA

* Correspondence and reprints
E-mail: piumi@biotec.jouy.inra.fr
Present address: Laboratoire de radiobiologie et d'étude du génome, Institut national de la recherche agronomique, Domaine de Vilvert, 78352 Jouy-en-Josas Cedex, France.

content, significantly larger in X-bearing spermatozoa, due to the size of the X chromosome (about 5% of the haploïd DNA content, while the Y contains only 1–2% of the cell DNA). Flow cytometry analysis revealed X/Y differences in DNA content ranging from 3.7 to 4% for boar, rabbit, bull and ram sperm cells [5]. Sperm sorting by flow cytometry was reported for the first time in 1987 for chinchilla [8]. Later, births were obtained using flow sorted spermatozoa in sheep [12], rabbits [9], pigs [6] and cattle [4]. Over 400 offspring have been born to this day using flow sorting technology. Before using such selected spermatozoa, the major requirement is the precise evaluation of the purity of sexed sperm samples. Different techniques may be used to carry out such an evaluation to avoid verification through the sex of offspring or embryos. These include the bovine sperm/hamster egg system [17], PCR analysis on individual sperm using X- or Y- specific primers [10], and fluorescent *in situ* hybridization (FISH), using X or Y specific probes. While the first technique is time-consuming, the second involves long extensive manipulations and extreme PCR conditions in order to amplify DNA from a single cell (although this drawback can be alleviated by amplifying repetitive DNA from the Y chromosome). The last technique is rapid and adapted for single cell analysis, at least on metaphase spreads. One drawback, however, is that it functions efficiently only with long DNA probes such as phage inserts, cosmids, bacterial artificial chromosomes (BACs) or yeast artificial chromosomes (YACs) or with DNA inserts that are shorter but repeated [13].

The aim of this study was to identify X or Y specific probes and to design techniques to assess the sex chromosome content of bovine spermatozoa. To detect the Y chromosome, the BRY4a probe was cloned. This 470 base pair (bp) DNA fragment is the bovine homologue of the ovine OY11.1 (Ovine Y chromosome region p11.1) and corresponds to a sequence highly repeated on the Y chromosome on three quarters of its length (Yp11-qter region) [19]. For X chromosome detection, the best tool available at the beginning of this study was a goat BAC library [18] of which the best FISH signal was obtained with a BAC containing microsatellite DVEPC076. The phylogenetic closeness of goats and cattle led us to hypothesize that at least some goat BACs would be able to specifically and efficiently recognize the bovine X chromosome and could be used for this purpose.

2. MATERIALS AND METHODS

2.1. Probes

2.1.1. Y chromosome detection

To obtain the 470 base pair (bp) fragment specific to BRY4a, PCR was performed in a mix containing 200 ng of male bovine genomic DNA, 2 mM of $MgCl_2$, 200 μM of each deoxynucleotide triphosphate (dNTP) (Pharmacia, Les Ulis, France), 0.5 μM of each primer [19],

5′ TGTACTTCATGTATTTAAAACAAAACAC 3′

and 5′ CAAGACCATACATATGTCATTATAGACAG 3′

and 2.5 U of Taq polymerase (Promega, Charbonnières, France). PCR conditions were 30 s at 94 °C, 30 s at 53 °C and 30 s at 72 °C during 30 cycles. Purity and concentration of the amplification product were evaluated on a 3% agarose gel.

After direct precipitation by ethanol, the fragment was cloned into a pGEM-T plasmid (Promega, Charbonnières, France) adapted to PCR products. This vector also confers resistance to ampicillin and allows blue/white color screening.

Conditions of ligation were established in order to get a 1:3 molar ratio of the vector/insert. Then a mix was made with 3 U of T4 DNA ligase (Promega, Charbonnières, France) in a final volume of 10 μL kept at 4 °C overnight.

Twenty μg of the ligation reaction (approximately 2 μL) was used to transform 50 μL of *E. Coli* JM109 competent bacteria (Promega, Charbonnières, France). The bacteria were spread on LB/agar plates (Sigma, St. Quentin Fallavier, France) containing ampicillin (100 $g \cdot L^{-1}$) IPTG (200 $g \cdot L^{-1}$) and X-gal (40 $g \cdot L^{-1}$) to get isolated colonies.

The following day, some white colonies were chosen and sub-cultured in 3 mL of LB medium (Sigma, St. Quentin Fallavier, France) containing 300 μg of ampicillin at 37 °C overnight under stirring. Plasmids were extracted by alkalin lysis ("miniprep") according to the protocol described by Birnboim and Doly [2] and inserts were sequenced with the ABI PRISM™ Dye Primer Cycle Sequencing Ready Reaction Kit and the ABI PRISM™ 377 DNA Sequencer (Perkin-Elmer, Courtaboeuf, France).

Labeling was performed by PCR using biotinylated deoxyuridine triphosphate nucleotides (biotin-dUTP). Efficiency was better using pUC/M13 forward and reverse sequencing primers flanking multiple cloning region. A 2:1 ratio of dTTP:Biotin-dUTP was used according to the manufacturer's manual (Boehringer Mannheim Biochemica, Nonradioactive In Situ Hybridization: Application manual). The mix composition was: 2 mM of $MgCl_2$, 0.1 mM of each dA-G-CTP, 0.06 mM dTTP, 0.04 mM Biotin-dUTP (Boehringer Mannheim, Meylan, France), 1 μM of each primer, 1 ng of plasmid DNA and 5 units of Taq in 100 μL. PCR conditions were 30 s at 94 °C, 30 s at 53 °C and 30 s at 72 °C during 30 cycles. Purity and concentration of the amplification product were evaluated on a 3% agarose gel.

2.1.2. X chromosome detection

The PCR library screening strategy and the primers used were described previously [16]. Briefly, the primers were designed from a mixture of $1 + X$ flow sorted bovine chromosomes [20]. After identification of the addresses in the library, clones were cultured, the BAC DNA was extracted and digested to evaluate insert size.

BACs were tested by FISH first in homologous condition on caprine metaphases to ensure X chromosome localization outside the pseudo-autosomic region (PAR: Xq42-43 in cattle) and secondly on bovine metaphases to check effective hybridization. Probes were labeled by Nick Translation using biotinylated dUTP (Biotin Nick Translation Mix, Boehringer Mannheim, Meylan,

France) and FISH protocol was carried out as previously described by Bahri-Darwich *et al.* [1]. Twenty microliters of labeled BRY4a amplicon or labeled BAC were added to 2 μL of carrier sonicated salmon sperm DNA (10 g $\cdot$ L^{-1} Sigma, St. Quentin Fallavier, France), 4 μL of competitor DNA (sonicated caprine genomic DNA, 1 g $\cdot$ L^{-1}, only for BACs because they contain repeated sequences), 350 μL of $AcNH_3$ 10 M, and 290 μL of Tris-EDTA 1/10. This solution was completed to 2 mL with cold ethanol. Tubes were placed at -80 °C for 20 min, centrifuged 10 min at 17 000 g, rinsed in 500 μL of 70% ethanol and again centrifuged 2 min at 17 000 g. Pellets were dried and dissolved in 20 μL of hybridization mix (formamide 5 mL, SSCP 2 mL).

2.2. Sperm decondensation

Bull sperm was obtained from a bull stud (Pierry, Marne, France). Two ejaculates from one bull collected at a 2 month interval were used. Each ejaculate was washed in 500 μL of PBS to a final concentration of 10^7 cells $\cdot$ mL^{-1}. Then spermatozoa were centrifuged at 1 000 rpm for 5 min and resuspended in 500 μL of CHAPS (Sigma, St. Quentin Fallavier, France) 4 mM in PBS (1.23 mg of CHAPS in 500 μL of PBS) for 10 min at ambient temperature. Cells were centrifuged at the same speed, washed once with PBS, centrifuged again then resuspended in 20 μL of PBS.

Due to its extreme compaction and highly condensed state, bull sperm chromatin must be swollen prior to hybridization. We used a protocol previously described by Zalensky *et al.* [23] which has been the most efficient in our hands. Decondensation was performed by adding 20 μL of PBS containing 20 mM dithiothreitol (DTT, Sigma, St. Quentin Fallavier, France) and 2 g $\cdot$ L^{-1} of heparin to the sperm cell solution for 30 min. Final concentration of DTT was 10 mM and heparin was 1 g $\cdot$ L^{-1}. After treatment, cells were dropped on slides, fixed with ethanol at room temperature and stored at -20 °C.

2.3. Hybridization and detection

FISH was performed essentially according to Bahri-Darwich *et al.* [1]. Probes such as DVEPC076 containing repeated sequences were denatured and incubated at 37 °C for at least 2 h 30 min before hybridization to let the competition process operate.

Biotinylated probes were detected by FITC-avidin (Vector, Valbiotech, Paris, France) diluted 1/400 in BN 1× containing 5% milk at 37 °C for 30 min. Then slides were washed 3 times in BN 1× at 42 °C each for 2 min.

Signals were amplified using biotinylated antiavidin (Vector, Valbiotech, Paris, France) diluted 1/100 in BN 1× containing 5% of goat serum (Vector, Valbiotech, Paris, France) for 30 min at room temperature and again with FITC-avidin at 37 °C for 30 min. Between incubations, slides were washed 3 times in BN 1× at 42 °C each for 2 min.

Counter-coloration was performed by adding 50 μL of a 5×10^{-3} g $\cdot$ L^{-1} propidium iodide (Sigma, St. Quentin Fallavier, France) solution in PBS to the slides. Ten microliters of antifade solution (Vectashield, Vector, Valbiotech, Paris, France) were then applied under coverslips.

Slides were screened using a 100× objective on a Leitz microscope equipped with propidium iodide and FITC band pass filters. Only individual and well-delineated sperm were scored.

3. RESULTS

3.1. Choice of the probes

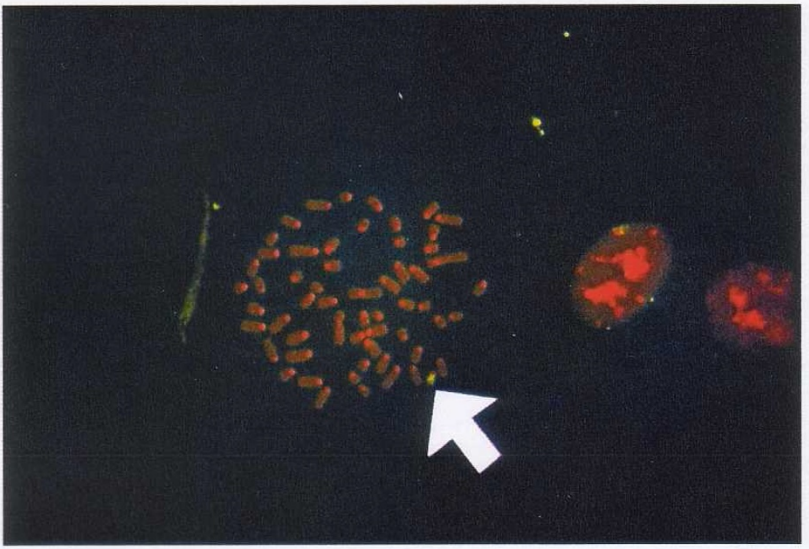
A BRY4a insert displaying a good similarity with the ovine counterpart was cloned after PCR amplification and used as a Y-specific DNA probe. Among four sequenced clones, BRY4a/2 showed fewer cloning errors, more homology with the OY11.1 ovine Y chromosome repeat region and finally was selected for FISH on spermatozoa. Results of hybridization on a bovine metaphase are shown in Figure 1a. For the X chromosome, our previous study [16] showed that three goat BACs out of the pseudoautosomal region yielded strong spots after hybridization on bovine metaphase chromosomes, DVEPC102, DVEPC041, the best being DVEPC076 located near the X centromere (BTA Xp11-13). This response (shown on Fig. 1b) led us to use this latter probe on bovine spermatozoa.

3.2. Hybridization results

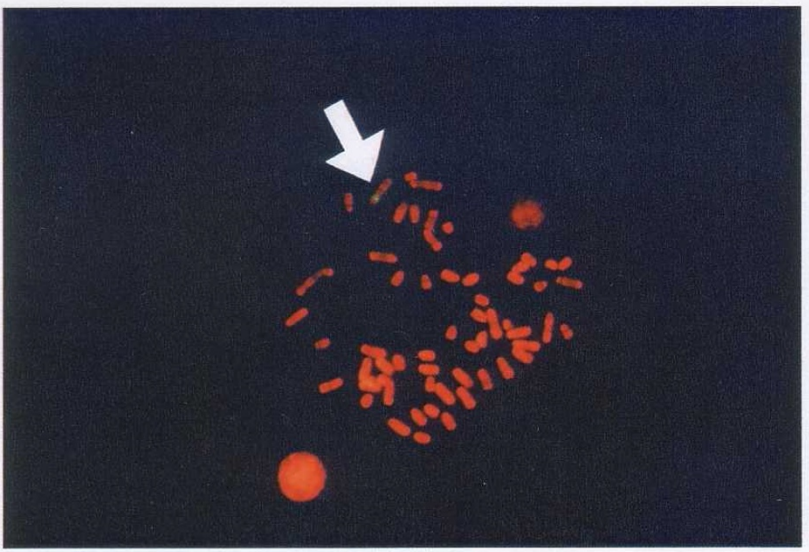
The sperm chromatin was decondensed by a heparin treatment [23] before hybridization. After hybridization with BRY4a, a green fluorescent signal was observed, located at or near the nucleus center (Fig. 1c), and was visible on 44.5% of the spermatozoa (n = 253, range 43–46%, 2 replicates (one for each ejaculate), Tab. I). DVEPC076 yielded a well-delineated green spot at the nucleus center (Fig. 1d) in 44.8% of the spermatozoa (n = 555, range 41–49%, 5 replicates (2 for the first ejaculate and 3 for the second ejaculate), Tab. I). These values were not statistically different from the expected 50/50 ratio (Chi squared test; P = 0.18 NS for BRY4a and P = 0.34 NS for DVEPC076).

4. DISCUSSION

Farming cattle necessitates the breeding of males or females for meat or dairy production, respectively. In this paper, specific gonosome probes were used to reliably identify bovine spermatozoa bearing chromosomes X- or Y in cattle. One of the major problems in FISH on spermatozoa is the extreme degree of condensation of the DNA, making access to specific hybridization sites very difficult. Different decondensation protocols have been established for different species. We started to work with a protocol derived from human sperm decondensation studies [3] which uses only DTT. We raised its concentration from 10 mM to 150 mM but decondensation of the sperm head was never as homogeneous as in humans. Then we used the protocol of Zalensky *et al.* [23] containing 1 $g \cdot L^{-1}$ of heparin in addition to DTT. Here this latter protocol gave us the best results. Heparin probably interacts with protamines and competes with DNA/protamine interactions. Heparin does not remove all the nucleus

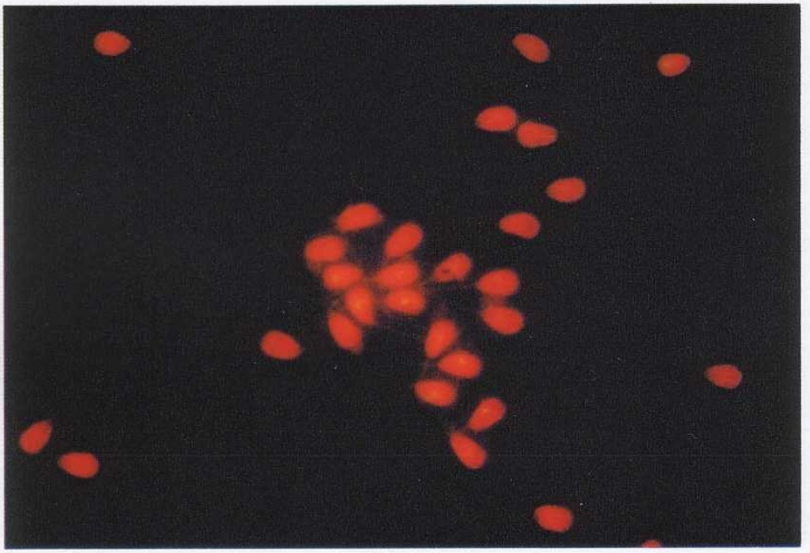


(a)

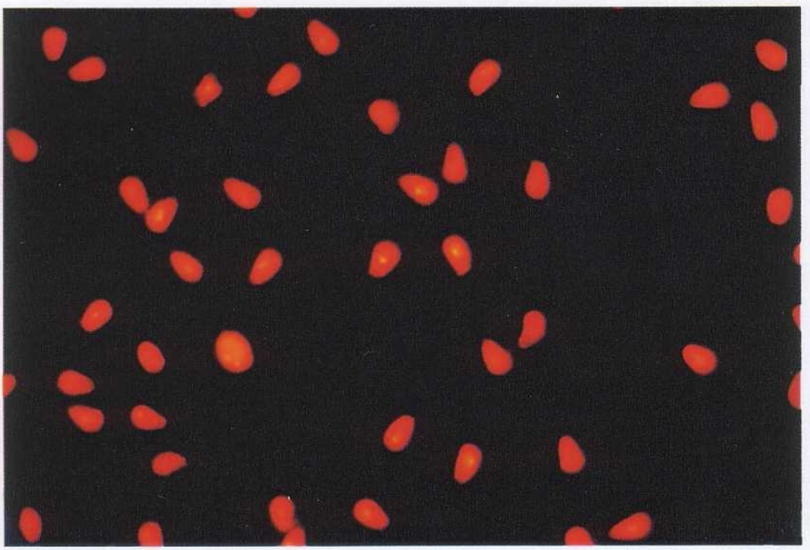


(b)

Figure 1. Fluorescent *in situ* hybridization using biotinylated probes detected by FITC-avidin (yellow/green spot). Total nuclear DNA was counterstained with propidium iodide (red). Bull sperm cells were swollen by heparin ($1\ g \cdot L^{-1}$) and DTT (10 mM). Magnification 10×100.
Figure 1a: Y-chromosome specific probe (BRY4a) on a bovine metaphase spread. Signal on the Y- chromosome is pointed by the yellow arrow.
Figure 1b: X-chromosome specific probe (DVEPC076) on a bovine metaphase spread. Signal on the Y- chromosome is pointed by the yellow arrow. *(continued on the next page)*



(c)



(d)

Figure 1. Continued.
Figure 1c: Y-chromosome specific probe (BRY4a) on bovine sperm cells.
Figure 1d: X-chromosome specific probe (DVEPC076) on bovine sperm cells.

protein and does not induce changes in nucleus morphology, making it possible to accurately control the evolution of chromatin decondensation. The heparin treatment was adapted to bovine cells since the optimal concentration varies according to the mammalian species.

Table I. Hybridization results.

Probe	Chromosome	Number of experiments	Labeled spermatozoa (%)	Counted spermatozoa
BRY4a (specific of 3/4 of the bovine Y chromosome)	Y	2 (1 of each ejaculate)	46	103
			43	150
			44.5[a]	**253**
DVEPC076 (specific of the bovine Xp11-13 chromosome)	X	5 (2 for the first ejaculate and 3 for the second ejaculate)	41	123
			47	99
			49	108
			42	115
			45	110
			44.8[a]	**555**

[a] Not statistically different from 50%.

Compared to other probes such as whole sorted chromosomes, our probes have the advantage of being cloned and, even though the labeling signal is finer, these probes should yield more reproducible results.

Progress in flow cytometry has made it possible to separate the two types of spermatozoa. Evaluating the quality of a sorting experiment is therefore an important step before proceeding to artificial insemination in this species. The probes that were developed labeled 90% of spermatozoa when considering both of them. This value is sufficient for evaluating the sorting of one type of spermatozoa, although some complementary experiments should be carried out on "sexed" sperm, frozen or not. The two developed probes can be used in two color labeling in order to distinguish X- and Y-bearing spermatozoa in the same experiment. In such experiments unlabelled sperm will not be counted in either category. The use of a confocal microscope could alternatively be envisaged in order to "search" the signal in a third dimensional axis in all spermatozoa. However, not all the spermatozoa were labeled. Different hypotheses can be formulated to explain these discrepancies, like failure to hybridize due to cell preparation, position of the chromosome inside the cell, or absence of the chromosome target (aneuploidy). To solve this question, the simultaneous use of the two probes labeled with different colors seems to be the most straightforward approach. The central localization of the signals may be partially explained by recent data on DNA packaging inside the sperm nucleus. In bulls, telomere-telomere dimeric associations are clustered at the periphery of mammalian sperm cells [22]. Furthermore, the same authors found that centromeres are located inside chromocenters across the entire width of the bovine sperm head equatorial region [15]. Nevertheless, chromosome localization in sperm can also depend on the predominant chromosome morphology (meta- acro- or telocentric) in a given species. Few data are available on chromosome position inside the sperm nucleus. In hamsters, Ward *et al.* [21] showed that genes belonging to the same chromosome seem to be located together in

the sperm head region, but with no preferential orientation of one gene relative to the other. They suggest a plasticity allowing a certain range of possible localizations. Recently, it has been shown in humans that there does not seem to be any correlation between the location of sex and autosomal chromosomes in spermatozoa [11]. Finally, it shows that interphase FISH data concerning physical mapping of chromosomes must be interpreted with much precaution.

The FISH technique and gonosomal probes proved to be effective in measuring the sex ratio in spermatozoa. This method therefore determines the efficiency of different techniques of sperm sorting such as flow cytometry. To increase this accuracy, these two probes labeled with two different colors will be used simultaneously on the same sperm cells in another set of manipulations.

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