An X–Y paint set and sperm FISH protocol that can be used for validation of cattle sperm separation procedures

W. Rens¹, F. Yang¹, G. Welch², S. Revell³, P. C. M. O'Brien¹,
N. Solanky¹, L. A. Johnson² and M. A. Ferguson Smith¹

¹Centre for Veterinary Science, Department of Clinical Veterinary Medicine, Veterinary Cytogenetics Group, University of Cambridge, Madingley Road, Cambridge CB3 0ES, UK;

²USDA, ARS, GGPL, B200, BARC-East, Beltsville, MD 20705, USA; and ³Genus Plc, Llanrhydd, Ruthin, Clwyd, UK

X and Y chromosome paints were developed from sorted yak chromosomes for sexing cattle spermatozoa. Clear hybridization signals were obtained for every spermatozoon using a modified sperm decondensation protocol and fluorescence *in situ* hybridization (FISH). The procedure was evaluated using the established Beltsville sperm sexing technology, which separates spermatozoa by flow cytometry into X- and Y-bearing fractions. Close agreement was found between the assessment of sperm separation by flow cytometry and by FISH with the X–Y paint set. The FISH method is a simple, reliable and robust procedure for assessing the effectiveness of separation of X and Y spermatozoa.

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Introduction

Determination of the sex of progeny at fertilization using sexed spermatozoa is now regarded as an important and realistic development in the cattle industry and one that could improve the economics and management of both dairy and beef breeder operations (Amann, 1999). The Beltsville sperm sexing technology is the only method that has proved effective for sexing viable spermatozoa (Johnson, 1995). It uses flow cytometry for sperm sorting and was started in the late 1980s (Johnson *et al.*, 1989), and has been modified considerably since then (Rens *et al.*, 1996, 1998, 1999; Johnson and Welch, 1999; Johnson *et al.*, 1999). Alternative procedures for artificial insemination have also resulted in improved efficiency (Johnson, 1995; Catt *et al.*, 1996; Rath *et al.*, 1997; Seidel *et al.*, 1997; Long *et al.*, 1998). Although sorting by flow cytometry is effective, different procedures for sorting X- and Y-bearing spermatozoa are being developed to obtain larger numbers of sexed spermatozoa inexpensively (Blecher, 1999; Hendriksen, 1999; Van Munster, 1999) and these endeavours would benefit from the availability of a robust method to validate the efficiency of separation of X and Y spermatozoa. Flow cytometric analysis is currently used to assess the effectiveness of sperm separation on the basis of DNA content (Welch and Johnson, 1999), but a flow cytometer is not always available and, more importantly, an experienced biologist is required to obtain reliable results. Application of the PCR method to individual sorted X and Y spermatozoa using sex-specific primers has been performed

successfully, but is time consuming (Welch *et al.*, 1995). Another possibility is the use of fluorescence *in situ* hybridization (FISH) to sex spermatozoa. Separate probes for X- and Y-bearing spermatozoa are required to avoid false negative results for FISH.

In the present study the development and use of sex chromosome-specific paints obtained by degenerate oligonucleotide-primed-PCR (DOP-PCR) amplification of flow sorted X and Y chromosomes from a yak cell line are described. The use of an optimized protocol for FISH on cattle spermatozoa resulted in clear signals in > 90% of spermatozoa. The fluorescence-labelled X- and Y-specific paints were tested on samples of cattle spermatozoa sorted by the established Beltsville sperm sexing technology. The results of the present study indicate that the FISH procedure is robust, reproducible and simple to use in evaluating the effectiveness of methods for sex sorting spermatozoa.

Materials and Methods

Paint production

A primary fibroblast cell culture was obtained from the skin of a yak (*Bos grunniens*). Cells were grown at 37°C in Dulbecco's modification of minimal essential medium (BRL) enriched with 15% fetal bovine serum (Gibco BRL, Paisley), penicillin (100 iu ml⁻¹; Gibco BRL), streptomycin (100 µg ml⁻¹; Gibco BRL) and glutamine (2 mmol l⁻¹; Gibco BRL). Chromosomes were isolated as described by Yang *et al.* (1995). The chromosomes were prepared for sorting by staining with 40 µg Chromomycin A3 ml⁻¹ (Sigma, Poole), 2 mmol MgSO₄ l⁻¹ and 2 µg Hoechst 33258 ml⁻¹ (Sigma) and incubated for at least 2 h. Ten minutes before flow

analysis, sodium sulphite and sodium citrate were added to final concentrations of 10 and 25 mmol l⁻¹, respectively. The stained chromosome preparations were sorted on a Facstar Plus flow sorter (Becton Dickinson, Erembodegem) equipped with two 5 W argon ion lasers. Four hundred X and Y chromosomes were sorted directly into separate 500 µl PCR tubes each containing 30 µl sterile distilled water. The flow sorted chromosomes were used as templates for amplification by DOP-PCR (Telenius *et al.*, 1992) using 2 µmol 6-MW l⁻¹ (5'-CCGACTCGAGNNNN-NNATGTGG-3' where N represents any base). Primary DOP-PCR products were used as a source of template for the incorporation of biotin-16-dUTP (Boehringer Mannheim, Lewes) or Cy3-dUTP (Amersham, Little Chalfont). Fifty nanogrammes of each of the X and Y paints was made up to 15 µl with hybridization buffer (50% (v/v) deionized formamide (BDH, Poole), 10% (w/v) dextran sulphate (Sigma), 2 × SSC and 0.5 mol phosphate buffer l⁻¹, pH 7.3).

Washing of spermatozoa

Unsorted fresh cattle spermatozoa were supplied in a Tris-based, ambient temperature extender and checked for concentration. Flow sorted cattle spermatozoa were supplied in a TEST-yolk medium (Johnson *et al.*, 1989) at a concentration of 1.5×10^6 spermatozoa ml⁻¹. Sperm samples were washed in a solution of 0.01 mol Tris l⁻¹ and 0.9% (w/v) NaCl, and were centrifuged at 400 g for 10 min to remove the supernatant. A final concentration of 2.5×10^8 spermatozoa ml⁻¹ was obtained by adding a solution of 0.01 mol Tris l⁻¹ and 0.9% (w/v) NaCl.

Decondensation of spermatozoa

The protocol for decondensation of the cattle spermatozoa is based on the method reported by Rodriguez *et al.* (1985). A volume of washed spermatozoa was put in a 0.5 ml Eppendorf tube and was frozen by immersion in liquid nitrogen and then thawed rapidly. An equal volume of 0.25 mol dithiothreitol l⁻¹ in 0.01 mol Tris l⁻¹ and 0.9% (w/v) NaCl was added and the tube was incubated for 2.5 min at room temperature. Subsequently, an equal volume of 1% (w/v) sodium lauryl sulphate and 1.9% (w/v) disodium tetraborate was added and incubated for 10 s, after which 70% (v/v) ethanol was added at ten times the total volume of the other reagents.

A droplet of 2 µl of decondensed spermatozoa was deposited on a slide. The slide was immediately dried at 50°C on a hot plate. The slide was dehydrated in 100% ethanol for 5 min and dried at room temperature. The spermatozoa should be checked at this stage with a phase-contrast microscope. The sperm tail should remain attached but deformed into a curled shape (Fig. 1c). If sperm tails are straight (Fig. 1a) or only slightly affected (Fig. 1b), the decondensation is not sufficient. If the sperm tail is absent (Fig. 1d,e), the decondensation has progressed too far. In both cases, the sample can be disposed of. Different

Fig. 1. (a) Insufficiently decondensed cattle spermatozoon with a straight tail. (b) Spermatozoon in which decondensation has just started. (c) A spermatozoon with a curly tail that will have clear hybridization signals. (d) Large and sometimes fragmented FISH signals are obtained with a spermatozoon where the tail was removed. (e) A progressively decondensed spermatozoon.

dilutions of the reagents with new volumes of washed spermatozoa should be used to obtain the correct decondensation, as this is more effective than changes in the duration of treatment.

Pepsin treatment (optional)

The slide was incubated in 0.01% (w/v) pepsin (Sigma) in 10 mmol HCl l⁻¹ for 30 min at room temperature. Subsequently, the slide was washed three times in 2 × SSC for 1 min and rinsed with distilled water. The slide was then dehydrated through an ethanol series: 2 min in 70% ethanol, 2 min in 90% ethanol, 5 min in 100% ethanol and air dried at room temperature.

Hybridization

The mix of yak X and Y chromosome paints was denatured for 10 min at 70°C and chilled on ice. The slide was baked at 65°C for 30 min, incubated for 20 min in 70% (v/v) formamide in 2 × SSC at 80°C to denature the cattle sperm DNA and quenched in ice cold 70% (v/v) ethanol for 5 min. The slide was dehydrated through a series of ethanol: 2 min in 70% ethanol, 2 min in 90% ethanol, 5 min in 100% ethanol and air dried at room temperature.

A 10 µl droplet of denatured chromosome paint was applied to the slide, covered with a glass coverslip and sealed with rubber solution. The slide was incubated overnight in a moist container at 37°C.

Detection

After incubation, the rubber solution and coverslip were removed and the slide was washed twice for 5 min in 50%

Fig. 2. (a) X-Y paints hybridized to a bovine metaphase. X-Y paints hybridized to three X-sorted samples indicating proportions of (b) 90, (c) 75 and (d) 50%. X-Y paints hybridized to three Y-sorted samples indicating proportions of (e) 90, (f) 90 and (g) 50%. Scale bar represents 10 μ m.

Table 1. X- and Y-labelling of bovine spermatozoa sorted by flow cytometry

Sample	Reanalysis		X-Y paint set (%)			Double-labelled	Number of spermatozoa counted
	X	Y	X	Y	Unlabelled		
Day 1							
1	8	92	3	89	8	—	112
2	50	50	46	50	4	—	104
3	87	13	76	19	5	—	139
4	50	50	47	47	6	—	107
5	91	9	85	5	10	—	87
6	18	82	12	82	4	2	74
Day 2							
1	5	95	3	93	4	—	100
2*	91	9	97	2	1	—	110
3	79	21	74	24	2	—	110
4	20	80	16	78	3	3	116

*Significant difference compared with value obtained with X-Y paint analysis ($P < 0.05$).

(v/v) formamide in $2 \times \text{SSC}$ at 43°C , twice for 5 min in $2 \times \text{SSC}$ at 43°C and three times for 3 min in $4 \times \text{SST}$ ($4 \times \text{SSC}$ containing 0.05% (v/v) Tween 20 (Sigma)) at 43°C . The biotin labelled X paint was visualized with avidin–fluorescein isothiocyanate (FITC) (1/500 in $4 \times \text{SST}$)/anti-avidin–FITC (1/250 in $4 \times \text{SST}$, Vector Laboratories, Burlingame) solution: 200 μl of this solution was deposited on the slide and covered with parafilm. The slide was incubated for 20 min at 37°C and washed three times for 3 min in $4 \times \text{SST}$ at 43°C . Sperm DNA was counterstained with 0.08 μg 4'6-diamidino-2-phenylindole ml^{-1} (DAPI; Sigma) solution in $2 \times \text{SSC}$ for 2 min and the slide was mounted in Citifluor antifade AF1 (Citifluor Ltd, London). Spermatozoa were observed with a Leica DMRXA fluorescence microscope equipped with an automated filter wheel with Cy3, FITC and DAPI specific filters (LEICA Microsystems). At least 100 spermatozoa were counted for each experiment. Images were captured using Leica QFISH with a cooled CCD camera (Photometrics Sensys) through $\times 40$ or $\times 63$ oil-immersion objectives.

Spermatozoa sorted by flow cytometry

On two separate days cattle sperm samples were sorted using the Beltsville sperm sexing technology in Beltsville, MD (Johnson, 1995). Spermatozoa were deliberately sorted with ratios of X:Y spermatozoa of 90:10, 10:90, 75:25, 25:75 and 50:50. The proportions within each sorted sample were assessed by flow cytometric reanalysis of an aliquot of each sample (Table 1). This aliquot was sonicated first to obtain sperm nuclei after which Hoechst 33342 was added to obtain uniform nuclear staining. Analysis of the aliquots produced histograms that were fitted to a double Gaussian curve to determine the proportions of X and Y populations. This procedure is an accurate assessment of the proportions of X and Y spermatozoa (Welch and Johnson, 1999). The sorted and unsorted samples (sperm concentration 1.5×10^6 spermatozoa ml^{-1}) were shipped on dry ice to Cambridge, UK. The samples were coded and analysed without knowledge of the flow cytometry results; these were revealed after the FISH results were obtained.

Statistical analysis

A single sample chi-squared test on raw data was used to compare fractions of X- or Y-labelled unsorted cattle spermatozoa with a 50:50 distribution. Differences between flow cytometry assessment and the FISH procedure were analysed by a 2×2 chi-squared test on raw data with Yates' correction.

Results

Decondensation of sperm DNA

The condition of the tails of the spermatozoa of *Bos taurus* was used as a measure of sperm expansion. When the tail of a spermatozoon is straight (Fig. 1a) or slightly

affected (Fig. 1b), the spermatozoon is not decondensed sufficiently and results in weak hybridization signals. Spermatozoa with curly tails (Fig. 1c) were regarded as having optimal treatment and had clear hybridization signals. Large and sometimes fragmented FISH signals were obtained from spermatozoa where the tail was absent (Fig. 1d) or with progressively decondensed cattle spermatozoa (Fig. 1e). It was noted, from the condition of the sperm tails, that the flow-sorted spermatozoa were more sensitive to the decondensation reagents. This finding was possibly due to the inevitable freezing and thawing process that the sperm samples underwent, as they were shipped on dry ice. A 5–10 times dilution of the decondensation reagents appeared to be optimal for these samples. All spermatozoa on an individual slide showed the same degree of decondensation when the sperm decondensation protocol was used.

X–Y labelling of metaphase chromosomes

Hybridizing the X–Y paint set to a cattle metaphase illustrates the precise specificity of the paints for their respective chromosomes (Fig. 2a).

X–Y labelling of non-sorted spermatozoa

Percentages of X chromosome bearing and Y chromosome bearing cattle spermatozoa were first determined in three samples containing non-sorted fresh spermatozoa by assessment with the X–Y paint set (Table 2). Statistical analysis showed that the percentages of X-painted spermatozoa and Y-painted spermatozoa were not significantly different from a 50:50 distribution.

X–Y labelling of spermatozoa sorted by flow cytometry

Hybridization results on different sorted cattle sperm samples are shown (Fig. 2b–g). Three different X-sorted purities (Fig. 2b–d) and three Y-sperm sorts (Fig. 2e–g) are shown. A comparison was made between the proportions of X and Y spermatozoa in the sorted samples as assessed by flow cytometry and by the X–Y paint set (Table 1). The use of the X–Y paints for assessment resulted in percentages in close agreement with the cytometric results. The statistical analysis showed that only sample 2 of day 2 had different assessments. However, both methods revealed that the proportions of X spermatozoa were $> 90\%$. Two samples showed a small percentage of haploid spermatozoa that were double labelled. In these instances, both X and Y signals were weak but still intense enough for them not to be counted as unlabelled; they may be instances of sex chromosome aneuploidy.

Discussion

In the present study the development of a chromosome paint set that distinguishes X spermatozoa from Y spermatozoa in cattle is described. Chromosome paints

Table 2. X- and Y-labelling of non-sorted bovine spermatozoa

Sample	X (%)	Y (%)	Unlabelled (%)	Number of spermatozoa counted
1	50	45	5	88
2	47	47	6	108
3	44	48	8	113

All samples show no significant difference from a 50:50 distribution.

were constructed for this purpose as they are highly target-specific and result in clear signals (Telenius *et al.*, 1992). However, at first the use of paints on decondensed cattle sperm was problematic and it was necessary to develop an improved sperm FISH protocol.

The DNA decondensation protocol described in the present study is the result of a search for a technique that does not degrade DNA, is quick and reproducible and leads to a level of decondensation similar for every cattle spermatozoon in the sample, thereby avoiding the need to search for an area on the slide with optimum sperm decondensation (Hassanane *et al.*, 1999). Exposing spermatozoa to the decondensation reagents while they were still in suspension in a test tube was found to give more rapid and reproducible results than did placing the spermatozoa on a slide.

Most protocols for sperm decondensation use a combination of dithiothreitol and lithium diiodosalicylate for human spermatozoa (Martin and Ko, 1995), or dithiothreitol only for pig spermatozoa (Kawarasaki *et al.*, 1996). Sperm enlargement was inadequate when bovine spermatozoa were exposed to these reagents. Dithiothreitol in combination with heparin was found to enlarge bovine sperm heads, but this is associated with a removal of the acrosome and release of DNA; these preparations gave poor FISH signals. Dithiothreitol with sodium lauryl sulphate and disodium tetraborate was found to be the most effective reagent for use on bull spermatozoa. This finding is in agreement with the report by Jager *et al.* (1990), in which bull spermatozoa were found to contain protamine I only, which is maximally crosslinked by disulphide bridges. Human spermatozoa contain protamine II as well as protamine I and are less stable than bull sperm nuclei. The dithiothreitol in the mixture is necessary to reduce disulphide bonds, whilst sodium lauryl sulphate, which is a charged detergent, precipitates protamines (Colom and Subirana, 1979). In the present study it was observed that disodium tetraborate was essential for keeping spermatozoa intact and that without this reagent, spermatozoa became speckled and balloon-shaped after decondensation.

The optimum sperm concentration found in the present study was 2.5×10^8 spermatozoa ml⁻¹. When lower concentrations were used, it was observed that dilution of the reagents rather than adjustment of the timings resulted in consistent levels of decondensation. Drying the slide

quickly on a hot plate, immediately after a decondensed sperm aliquot was applied to it, prevented any further decondensation, which may occur during a slower drying period.

In the present study the sperm preparations were observed by phase-contrast microscopy before FISH to check their decondensation status. The condition of the sperm tail proved to be a good indicator for satisfactory hybridization, at least in cattle. When the tail was straight, signals were weak. When the tail was disintegrated, the hybridization signal was large and sometimes fragmented. Optimum results were obtained when the tail was curly.

It was necessary to obtain bovine-specific sex chromosome paints by amplification of flow-sorted yak chromosomes as the X and Y chromosomes of cattle were not represented by individual peaks in the flow karyotype of the available cattle cell line. The yak X and Y chromosome paints were specific for the cattle X and Y chromosomes. These paints also gave clear signals when hybridized to cattle interphase nuclei (image not shown).

The X–Y paint set was hybridized to non-sorted and sorted spermatozoa to evaluate its use for the sexing of cattle spermatozoa. In both experiments, > 90% of the spermatozoa observed showed a clear hybridization signal. Labelling of the non-sorted spermatozoa showed no significant difference with a 50:50 distribution. The experiments with sorted spermatozoa showed a close agreement between the FISH assessment and the assessment by flow cytometry. Spermatozoa were deliberately sorted with fractions near 90, 75 and 50% for both X and Y spermatozoa and were correctly assessed as such, showing that it was possible to obtain a quantitative evaluation by FISH. The ability to differentiate between 50% and 70% for sex-specific spermatozoa is adequate to show that the method under development has potential for sperm sexing. Validation of the sperm sex ratio by flow cytometry has been used for the development and implementation of the Beltsville sperm sexing technology. The FISH procedure described here, using an X–Y paint set derived from the yak (available from Cambio Ltd, Cambridge), should prove useful in evaluating and monitoring future X–Y sperm separation methods in *Bos taurus*, and probably in many other species of Bovidae also.

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