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**Detection of the X- and Y-chromosomes of different Bovinae species by
fluorescence in situ hybridization, studied in the context of their evolution and
separation of X- and Y-bearing spermatozoa**

Thesis

written by

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Introduction

Being able to preselect the sex of offspring at the time of conception ranks among the most sought-after reproductive technologies of all time. This ability runs counter to trends of nature but carries great importance in human medicine and animal breeding, especially in cattle.

In humans, pre-conception selection of the sex of offspring can serve to prevent the manifestation of sex-linked diseases, thus allowing for carrier mothers to have healthy daughter.

The use of sex pre-selection in today's livestock management scheme can accomplish several economical and biological goals. Cows are preferred in the dairy industry for milk production, and to become mothers of the next generation. An important future issue can be the production of drugs or enzymes by transgenic animals using specific expression in the mammary glands. Males are the wanted sex for beef production due to the more efficient growth properties.

Numerous attempts were made to achieve this goal during the last century. For practical reasons the separation of spermatozoa is superior to embryo sexing, because it can easily fit into the artificial insemination based cattle breeding schemes. Most of the methods are based on the theoretical differences of X- and Y-chromosome bearing spermatozoa, which are originated from the existing difference in the size, thus in DNA content of the two chromosomes (3.5-4%). This difference was measured by flow cytometry, which represents the technical bases of the only successful semen separation method. X- and Y-bearing spermatozoa could be separated after staining the total DNA content with a UV excitable dye and converting the detected signal difference into surface charge difference. Positive and negative cells are separated into collection tubes by a cell sorter. Although thousands of different animals were born inseminating semen separated by flow cytometry, there is still concern about its genetic safety due to the possible harmful effect of the applied DNA staining, UV detection and electrical separation. All other methods tend to use the proposed differences in physical, or special surface properties (dry mass, specific gravity, swimming velocity, charge, protein profile, antigens) have resulted contradictory findings ranging from great success to complete failure.

The background of these conflicting results may be found in the difficulty of objective determination of difference in the investigated property and the lack of sensitive and high throughput test method whereby the separation is verified. Recently, fluorescence in situ hybridization (FISH) is the method-of-choice considering the requirements mentioned above.

A second expectation of a reliable sexing method is to produce not only sex-determined, but highly viable fractions of semen.

Scope of the thesis

Based on these I aimed to

- adapt an efficient and precise method to control the sex-ratio of a cattle semen sample
- test the fluorescence in situ hybridization method on other related domestic mammals (yak – *Bos /Poephagus/grunniens*, L.; water buffalo – *Bubalus bubalis*, L.), where genetic improvement could be achieved by sexed semen, but the recent status of these experiments are lagged behind those in the cattle species. The detection of the sex chromosomes of these species provides the possibility to reveal evolutionary conservations and/or differences, as well.
- develop a combined method for the simultaneous evaluation of viability and separation efficiency in a sample processed by any approach.
- perform an experiment to measure and compare the head surface of dead, live and X- or Y-chromosome bearing bull spermatozoa.

Applied methods

- Cattle, yak and buffalo metaphase chromosome preparations were made according to standard cytogenetic techniques. Cultures of peripheral blood lymphocytes were set up in RPMI 1640 medium containing 20% fetal calf serum and pokeweed mitogen. Before harvesting on the third day of culture, the cells were treated with colcemid and swollen in a hypotonic solution.
- The locus specific cattle BC1.2 hybridization probe was prepared by polymerase chain reaction (PCR). Bovine male genomic DNA, isolated from blood served as template. The PCR product was labeled with biotin-16-dUTP during the same reaction.
- The whole X- and Y-chromosome specific painting probes were made from flow sorted yak chromosomes, amplified and labeled with biotin and Cy3, respectively by DOP-PCR (Degenerate Oligonucleotide Priming-PCR)
- Fluorescence in situ hybridization was used to detect the BC1.2 probe or the X-Y paint set on chromosomes and spermatozoa. Decondensation of sperm chromatin was carried out by papain digestion. Biotinylated signals were visualized using layers of avidin-FITC (fluorescein-isothiocyanate) or alternatively by the Tyramide Signal Amplification System where the fluorescent signal was detected by sequential application of streptavidin-HRP (streptavidin-horseradish peroxidase) and fluorescein-tyramide solutions. The Cy3-labeled Y-chromosome specific painting probe was detected directly in microscope through the rhodamine filter. The cells were counterstained with DAPI (4,6-diamino-2-phenylindole) and mounted with Vectashield. The preparations were investigated using a Zeiss Opton or an Olympus BX-60 fluorescence microscope. The latter was

equipped with a Cohu B/W CCD camera. Pictures of representative areas were taken using a conventional microscope camera or by the ISIS software. Head area measurements were performed on pictures taken after the viability and acrosome staining by the Scion Image Beta 4.0.2. image analysis software.

- For statistical analysis the Chi-square test was applied to reveal variations between the animals investigated and deviation of frequency of total X- and Y-signals from the expected 50:50 distribution. The data from the head area measurements were analysed using the Statistica 5.5 software for descriptive statistics and ANOVA.

Results and Discussion

To apply FISH for sexing spermatozoa the two major problems are to find or prepare an appropriate sex-chromosome specific hybridization probe, and the efficient detection of signals in the compact sperm chromatin. Since no commercial probe was available for the sex chromosomes of large domestic animals until the middle of 2002, groups interesting in sexing of spermatozoa used clones containing large DNA fragments, probes generated by the special technique of chromosome microdissection and DOP-PCR or small-sized repetitive probes for the Y-chromosome. Although the successful national experiments using the first two approach in cooperation with foreign laboratories, the difficulty and cost of continuous cell propagation, clone preparation and labeling in the case of the first strategy, and the lack of appropriate micromanipulator, other equipments and extremely laborous preparation of the probe by the second way, limited to use them routinely in Hungary.

I have adapted a simple but efficient FISH procedure using a cattle repetitive Y-chromosome fragment, specific for the BC1.2 locus. The optimization of the probe-preparing polymerase chain reaction by modification of the $MgCl_2$ concentration provides the possibility of the amplification and labeling of the probe in a single step without the need for further purification. The hybridization conditions and detection methods were also optimized and the technique was used to visualize the cattle Y-chromosome on metaphase chromosomes and in spermatozoa. We compared two different ways to detect the specific probe-target contact. Using the Zeiss Opton microscope and without the possibility of digital image processing, the conventional immunological detection method (biotin/avidin-FITC) was not enough sensitive for fast and effective evaluation. The tyramide signal amplification system is based on the generation of reactive tyramide molecules by the horseradish peroxidase (HRP), bound to the biotin through avidin. These molecules react fast in the microscopic local area of the incorporated biotin and label the tissue with the carried fluorochrome. This system gave increased signal intensity and reduced detection time, so it allows to count a sufficient number of cells for the analysis in a short time and a simple way.

Based on the morphologically similar sex chromosomes, I applied the bovine Y-specific probe for sexing yak spermatozoa. The goal was to elaborate a rapid and effective *in vitro* technique to control the ongoing experiments (foreign laboratories) on producing sex-oriented yak semen.

This probe, together with the only commercially available probe set for the detection of cattle X- and Y-chromosomes was applied on water buffalo specimens. In the case of this species, the use of sexed semen would represent an exceptional tool in some countries, like Italy, since the almost exclusive usage of buffaloes is milk and cheese production. The statistical analysis proved the reliability of both methods for sexing water buffalo spermatozoa. Hybridization with the yak X-Y paint set resulted in clear single signals in more than 92% (46.8% X and 45.8% Y) of total spermatozoa investigated from five bulls. Using the Y-chromosome specific BC1.2 probe, a clear hybridization signal was detected in more than 48% of the cells. The evaluation of the sex ratio of a given sample is, however, more reliable using the X-Y paint set as compared to the single Y-probe, since the former evaluation should provide information about sex chromosomes in all spermatozoa. By use of the single Y probe, information was restricted to the Y-bearing population of the cells analysed. If no X-chromosome marker is available, the use of an autosomal microsatellite probe, serving as an internal control of FISH efficiency, might improve the evaluation. Compared to the expensive, commercially available X-Y probe set the advantage of the PCR generated locus specific probe is the simple one-step preparation which is cost-effective and fast.

The decondensation of spermatozoa is necessary for efficient hybridization. I used papain digestion on the fixed microscopic preparations for this purpose. Differences were found between the three species in the optimal duration of treatment. To reach the approximate double-sized swelling of spermatozoa an average 5-min treatment was optimal for cattle spermatozoa. Water buffalo spermatozoa showed increased sensitivity (1-3 min) and for the decondensation of yak cells longer treatment was required (15-25 min). This may represent species specificity, however an increase of the duration of treatment was detected for older buffalo preparations suggesting the most important effect of age and storage conditions of slides.

Apart from the fact that the investigations mentioned above represent the first application of FISH for sexing yak and water buffalo spermatozoa, the detection of the probes on metaphase chromosomes provides insight into the evolutionary changes and conservation of the sex chromosomes of these Bovinae species. The great similarity of cattle and yak sex chromosomes was suggested by conventional cytogenetic investigations. At the molecular level the BC1.2 probe was the first verification of this conservation, although the subsequent use of the total yak chromosome painting probes on cattle sex chromosomes gave further evidence. The BC1.2 probe labeled specifically the telomeric region of the acrocentric water buffalo Y-chromosome. Comparison of this location to the place on the short arm of cattle Y-chromosome (Yp13-12) confirms that a pericentric inversion took

place in the evolutionary rearrangements between these species, which was suggested by conventional chromosome banding techniques. The X-Y paint set gave strong signals on the water buffalo sex chromosomes, but none of them labeled the centromeres. This might be explained by the rapid evolutionary changes of these sequences, and/or the loss of centromeric DNA as suggested by banding studies.

Although FISH can detect the separation of spermatozoa, an interesting question is the effect of the given separation method on cell viability. Development of a method for the combined evaluation of live/dead status and sex chromosome content can be a valuable new tool supporting this research field. Sequential application of trypan blue/Giemsa staining and sex-chromosome specific FISH have realized the above mentioned issue. Viability staining distinguishes cells according to their different membrane permeability at the moment of smearing. Trypan blue stains permeable 'dead' cells but not impermeable 'live' cells; it also distinguishes the permeability status of the sperm tail membrane. Giemsa-stained intact acrosomes are purple; double-stained (trypan blue plus Giemsa) loose acrosomes are dark lavender. Due to the trypan blue and Giemsa staining, the decondensation by papain is specific for the live cells, if a longer treatment was applied at an increased temperature (1 h, 40°C). After FISH the swelled live cells show a clearly distinguishable dark violet DAPI staining as compared to the light blue colour of dead cells which kept their original size. Differential decondensation of live vs dead spermatozoa caused the differences in the hybridization efficiency. While it was more than 95% in live cells, dead ones show signals in only 25%, due to the absence of decondensation. Significantly more Y-sperm were detected by FISH in the dead population, but it can be explained by the better labeling characteristic of the Y-chromosome probe consisted of mostly repetitive sequences.

The problems associated with the development of alternative separation techniques to flow cytometry are based on the lack of objective data on dimensions of spermatozoa. The knowledge of those values would provide the possibility to recalculate old theories and plan more convenient experiments. Using sequential steps of viability and acrosome staining and X-Y FISH I could categorize the cells according to morphology, membrane integrity, acrosome status and sex-chromosome content. This represents a unique opportunity to assign head surface size values to individual cells and at the same time to collect data from thousands of cells necessary for the statistical analysis. The head areas of more than 3000 spermatozoa of five bulls were measured. In all bulls, morphologically normal, viable cells with intact acrosome were significantly smaller than the dead cells with damaged acrosome ($41.52 \pm 2.56 \mu\text{m}^2$, $36.17 \pm 1.99\mu\text{m}^2$, respectively, $p < 0.001$). No significant difference in the head area between X- and Y-chromosome bearing viable, acrosome-intact spermatozoa was found in the individual bulls ($36.39 \pm 1.99 \mu\text{m}^2$ and $36.23 \pm 1.95 \mu\text{m}^2$, respectively, $p > 0.5$). However, significant between-bull differences were detected in all cell categories ($p < 0.001$).

New scientific results

- 1.** I used FISH for sexing yak spermatozoa for the first time. The specific localization of cattle BC1.2 sequence on the yak Y-chromosome proved the molecular homology of the two chromosomes.
- 2.** I used the cattle Y-specific DNA fragment (BC1.2) and an X-Y whole chromosome paint set to label the water buffalo sex chromosomes on metaphase spreads and in spermatozoa for the first time. I proved by statistical analysis that both methods are reliable to assess the separation of X and Y bearing water buffalo spermatozoa.
- 3.** The detection of the cattle BC1.2 probe on the water buffalo Y-chromosome gave the first molecular confirmation that a pericentric inversion took place in the evolutionary rearrangements between these species as suggested by banding studies. The absence of signals on the centromeres of the sex chromosomes using the X-Y paint set might be explained by the rapid evolutionary changes of these sequences, and/or the loss of centromeric DNA as suggested by banding studies.
- 4.** I have developed a combined method for the simultaneous evaluation of viability and efficiency of separation of X- and Y-spermatozoa using consecutive trypan blue and Giemsa staining, differential decondensation by papain and X-Y FISH..
- 5.** I measured the head surface areas of dead, live, and live X- or Y-bearing bovine spermatozoa for the first time getting information on the same cells. I found no difference between the size of X- and Y-bearing live spermatozoa, however dead cells were significantly enlarged by 15%.

Future applications

1. The sexing methods developed for yak or water buffalo species can support the basic research on development of semen separation methods.
2. Using the simultaneous evaluation of the separation efficiency and caused cell damage can further improve the separation experiments. Step-by step testing of the flow cytometry sorting method can reveal the bottlenecks of the most important separation technique.
3. We have only minor knowledge about sex chromosome aneuploidy on domestic mammals. The detection of the X- and Y- chromosomes on high numbers of animals and cells by FISH can serve this aim.
4. Using the head size values of different cell types we can evaluate the theoretical bases of alternative separation methods, other than flow cytometry and sorting.

List of publications

Papers published in international journals:

1. Révay T, Kovács A, Rens W, Gustavsson I
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X- and Y-chromosome bearing bovine spermatozoa do not differ in head area: a parallel image analysis/Fluorescence In Situ Hybridization study
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Poster at Hungarian conference:

17. Révay Tamás, P. Tardy Erika, Kovács András, Szalai Gábor, Tóth András, Salgó András
Szarvasmarha ondósejtek szexálása fluoreszcens *in situ* hibridizációval.
6. Szaporodásbiológiai Találkozó, Balatonfüred, 1999. 10. 25-26.