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Sex ratio determination in bovine semen: A new approach by quantitative real time PCR

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Abstract

Sex preselection of livestock offspring in cattle represents, nowadays, a big potential for genetic improvement and market demand satisfaction.

Sperm sorting by flow cytometer provides a powerful tool for artificial insemination and production of predefined sexed embryos but, an accurate verification of the yield of sperm separation remains essential for a field application of this technique or for improvement and validation of other related semen sexing technologies.

In this work a new method for the determination of the proportion of X- and Y-bearing spermatozoa in bovine semen sample was developed by real time PCR. Two sets of primers and internal TaqMan probes were designed on specific X- and Y-chromosome genes. To allow a direct quantification, a standard reference was established using two plasmid cDNA clones (ratio 1:1) for the specific gene targets. The method was validated by a series of accuracy, repeatability and reproducibility assays and by testing two sets of sorted and unsorted semen samples. A high degree of accuracy (98.9%), repeatability (CV = 2.58%) and reproducibility (CV = 2.57%) was shown.

The results of X- and Y-sorted semen samples analysed by real time PCR and by flow cytometric reanalysis showed no significant difference ($P > 0.05$). The evaluation of X-chromosome bearing sperms content in unsorted samples showed an average of $51.11 \pm 0.56\%$ for ejaculates and $50.17 \pm 0.58\%$ for the commercial semen. This new method for quantification of the sexual chromosome content in spermatozoa demonstrated to be rapid and reliable, providing a valid support to the sperm sexing technologies.

Keywords: Sex ratio; Bovine; Ejaculate; Real time PCR; Sexed semen

1. Introduction

Development of the reproductive technologies has made them possible to determine the sex of the embryos by Y and X chromosomal DNA sequences and to separate accurately X- and Y-chromosome bearing sperms into different populations. Both approaches can be useful in cattle breeding programs, leading to the possibility to plan matings for a specific sex.

Semen sexing, involving the separation of X- from Y-chromosome bearing sperms, implies its application in artificial insemination (AI) or in *in vitro* fertilisation (IVF) with the subsequent embryo transfer (ET). At present, the only proven method for producing a significant enrichment of X- and Y-chromosome bearing spermatozoa in mammals is the cell sorting by flow cytometry, based on DNA difference [1].

At the same time, the evolution of this important technology for the separation of spermatozoa by sexual

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chromosomes, required the development of accurate methods to verify the purity of the sexed sperms. Usually, sexed semen purity is verified by flow cytometry reanalysis [2] but the validation of data, by a reliable method which does not rely on the same instrumentation, is essential for the practical use or the improvement of such technology.

Since 1970, different techniques have been attempted to evaluate the sex ratio in ejaculates of different mammals. The interest for this kind of evaluation arose from the evidence that the proportion of male to female offspring in a given population (secondary sex ratio calculated at birth) could vary significantly from the expected 1:1 ratio [3]. The bases of this imbalance are still unclear but some studies suggested the environmental and physiological factors [4] or the time of insemination during estrus [5,6], as well as a distortion of the proportion of X- and Y-chromosome bearing spermatozoa in the semen [7–9], as possible causes of this disequilibrium. To investigate this last hypothesis, different techniques, such as the Quinacrine mustard staining for Y-chromosome bearing sperms identification [10], the Quantitative Southern Blotting [11], the semi-quantitative PCR for Y- and X-chromosome detection, eventually associated with autoradiography analysis [12,13,8,9], the PCR analysis on single spermatozoa [14,7] and the capillary electrophoresis [21] have been developed. Nevertheless, all these kinds of approaches were often approximate and/or time-consuming. Nowadays, the most reliable methods for the evaluation of the percentage of sexual chromosomes in spermatozoa, is the multicolour fluorescence in situ hybridisation (FISH) [15–20].

The present study is focused on the development, validation and application of a new method by TaqMan real time PCR to establish the frequencies of X- and Y-chromosome-bearing spermatozoa in bovine semen samples.

2. Materials and methods

2.1. Primers and probes design

Bovine Y- and X-chromosome specific primers and probes were selected using Primer Express software (PE Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA, USA), according to the parameters required for the real time PCR applications.

The Y-specific primers pair (forward: 5′-CCACGTCAAGCGACCCAT-3′ and reverse: 3′-CGAAGACGAAAGGTGGCTCT-5′) and the internal probe (5′-AACGCCTTCATTGTGTGGTCTCGTGA-3′) were designed on a conserved region of the bovine Y-chromosome-linked SRY gene that is responsible for male sex determination [22]. The Y-product amplification length was 66 pb (GenBank accession no. AF148462).

Oligonucleotide X-specific primers were designed to amplify a 96 bp DNA fragment on the intron 2 region of the bovine proteolipid protein gene (PLP) [23] (GenBank accession no. AJ222799). The sequences of the forward and reverse primers were, respectively: 5′-GTTGTGTTAGTTTCTGCTGTACAATAAAGTG-3′ and: 3′-TCCTACCCTCACCTGCCATC-5′. The internal probe sequence was: 5′-TGTATACACATAGCCCCTCCCTCTTGGACC-3′.

The two probes were labelled using FAM fluorochrome.

Prior to use, primers specificity was verified by Blast Software (www.ncbi.nlm.nih.gov) and the purity of the two amplification products were evaluated on 3% agarose gel.

2.2. Reference plasmids cDNA clone preparation

To gain an accurate prediction of the X and Y chromosome content in semen samples, a standard reference was prepared to obtain two regression equations. To this aim, the X and Y amplification products were fractionated by electrophoresis through a 3% agarose gel and then extracted and purified by QIAEX II Kit (Qiagen S.p.A, Milan, Italy). The DNA amplicons were sent to a specialised laboratory (Primm srl-San Raffaele Biomedical Science Park, Milan, Italy) for the manufacturing of the references, which consisted of a mixture (ratio 1:1) of two cDNA plasmids pCR 2.1 (Invitrogen S.R.L, Milan, Italy), produced, respectively, by cloning X- and Y-amplicons. Five scalar 10-fold serial dilutions (CO: 100–0.01%) of the plasmids mixture were performed over the range of copy numbers that include the amount of target DNA expected in the experimental samples. The initial concentration of each plasmid consisted of about 10^6 molecules.

2.3. Quantitative real time PCR

Real time PCR was performed by ABI PRISM 5700 Sequence Detection System (PE Applied Biosystems) which monitored the PCR reactions, for X and Y chromosome targets of a given DNA semen sample, through two fluorescence amplification plots (Ampl-X and Ampl-Y) (Fig. 1). The quantification was performed by experimental determination of the