Introducing and validation of SYBR Green Real-Time PCR method to determinate sex ratio in bovine semen

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\textbf{A B S T R A C T}

Flow cytometry is a widely used application for validating the accuracy of sperm sexing. However, this method is relatively expensive and requires considerable technical support. An alternative method employing simpler technology at low cost could be suitable for the evaluation of bovine semen in laboratories with low budgets. We used a SYBR Green Real-Time PCR assay to determinate sex ratio in bovine semen. The PLP and SRY genes were amplified to isolate the specific fragments of X- and Y-chromosome sequences, respectively. Two certified standard curves were obtained using two plasmids containing PLP and SRY amplicons. Our results show no significant difference in semen sex ratio in unsorted semen (54.7 ± 0.52% X and 47.6 ± 0.60% Y). However, significant difference was observed in X-sorted semen (93.3 ± 0.08% X and 91.4 ± 0.06% Y-sperm), as compared to the expected ratio in unsorted semen or the post-sorting reanalysis data. The evolution of X-chromosome bearing sperm content in unsorted samples showed an average of 52.6 for ejaculates and 51.8 for the commercial semen. In order to confirm our results, the accuracy, repeatability and reproducibility of the method were tested resulting in 98.2% accuracy, repeatability of CV = 5.59% and reproducibility of CV = 5.40%. Thus, this method is demonstrated to be a reliable and inexpensive way to test sexual chromosome content in semen samples.

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1. Introduction

Sorting X:Y ratio in semen is of the particular interest to many reproductive disciplines to evaluate the role of sex chromosomes in sex ratio of sperm, or to determine the success of semen sexing procedures (Parati et al., 2006). Controlling the gender of progeny, in both humans and production animals is a major focus of reproductive technology. Predetermination of sex in human offspring could be valuable in preventing the occurrence of sex-linked diseases particularly when medically indicated (Parati et al., 2006).

Shifting the sex ratio of a sperm population into X- or Y-bearing samples represents an effective approach to produce embryos of a predefined sex (Parati et al., 2006). The specific production of male or female offspring by sexing of sperm and artificial insemination (AI) is a long-standing goal in breeding of cattle and other mammals. It might considerably accelerate the attainment of breeding goals. The use of sexed semen in livestock relies on the sorting purity of sperm cells.

Focus of the previous studies has been to evaluate sex ratio determination in semen of different mammals. Interest in this kind of evaluation arose from evidence which demonstrates significant deviation from the expected ratio (Clutton-Brock and Iason, 1986; Parati et al., 2006).
Several techniques have been developed such as Quinacrine mustard staining for Y-chromosome bearing semen identification (Ogawa et al., 1988), quantitative Southern Blotting (Beckett et al., 1989), semi-quantitative PCR for Y- and X-chromosome detection, associated with autoradiography analysis (Lobel et al., 1993; Chandler et al., 1998, 2002, 2007), and capillary electrophoresis (Checa et al., 2002). Nevertheless, these methods are often approximate and/or time-consuming (Parati et al., 2006).

Most studies, especially in bovine, sex ratio determination was not only proposed for semen sexing, but could also provide an alternative method for flow cytometry reanalysis to confirm bovine sexed semen. Usually, the purity of sorted semen is determined by sort reanalysis, in which sorted sperm were reanalyzed using flow cytometry technology (Parati et al., 2006). Although this kind of evaluation is convenient and accurate it uses the instrumentation and associated errors that have contributed to poorly sorted semen. Sort reanalysis requires a relatively large number of sperm (Colley et al., 2008). Recently, in order to find an alternative method for sort reanalysis, two major approaches have been reported: single and pooled semen sex ratio determinations.

Fluorescent in situ hybridization (FISH) is a widely applicable method with successful hybridization signals for single sperm sex ratio determination (Schwerin et al., 1991; Kawarasaki et al., 1998; Piumi et al., 2001; Rens et al., 2001; Di Berardino et al., 2004; Habermann et al., 2005; Yan et al., 2006). Recently, Colley et al. (2008) developed a duplex PCR method, based amelogenin gene specific primers. Importantly, these two method (FISH and duplex PCR) have great potential to determine single sperm sex ratio determination when sperm number is low or sperm are very valuable. One of the most noteworthy methods used to evaluate sex ratio in pooled sperm is Real-Time PCR assay because of its sensitivity and simplicity. The first study was done by Joerg et al. (2004), and subsequently Parati et al. (2006) using TaqMan probes to the X- and Y-chromosomes for proteolipid protein gene (PLP), and male sex determination gene (SRY).

Real-Time PCR analysis, based on fluorescence DNA-dyes such as SYBR-Green or TaqMan probes can provide an independent assessment of sorting purity. The present study is focused on development and validation of quantitative SYBR Green Real-Time PCR method to determine sex ratio in bovine semen.

2. Materials and methods

2.1. Primer design

A pair of specific primers were designed for Bovine Y- and X-chromosome partial sequence using primer Premier5 (Premier Premier V.5; Premier Biosoft International, Palo Alto, CA, USA), according to the parameters required for the SYBR Green Real-Time PCR (Dorak, 2006).

The Y-specific primers pair (forward: GAACGAAGAGCGAAAGTGCGCT and reverse: GCGGTTAGAAGACTCCCTC) were designed on a conserved region of the bovine chromosome linked SRY gene. The Y-product amplification length was 120 bp (GenBank accession no. HQ908797).

X-specific primers were designed to amplify a 149 bp DNA fragment on the bovine proteolipid protein gene (PLP) (GenBank accession no. HQ875721). The sequences of the forward and reverse primers were, respectively: TAAAGGTGGTAGTTCAAGG and GCTGTGTAATACGGTGTAG.

Primers specificity was verified using genomic DNA (5 ng/reaction) extracted from the blood of male and female sheep, cattle, bull and human using Accuprep® Genomic DNA Extraction Kit (Bioneer Co., Daejeon, Korea). Purity of amplification products of X- and Y-chromosome were evaluated on 1.5% agarose gel (data not shown).

2.2. Standard curves preparation

To produce a standard curve, X and Y PCR amplions were subcloned into pTZ57R/T vector by TA cloning kit (Ins T/TA clone™ PCR Product Cloning Kit, MBI Fermentas Inc., USA). Cloned sequences were confirmed by standard Sanger sequencing. Five scalar 10-fold serial dilutions (CO: 100–0.01%) of every recombinant plasmid was 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 consists of 10^6 molecules.

2.3. Semen samples

Two different sets of sorted and unsorted bovine semen samples were analyzed by Real-Time PCR. The first set was composed of 50 straws of unsorted semen samples that were collected from 50 different Holstein bulls with proven fertility. The second set was composed of 8 straws of sorted samples for Y-chromosome and 8 straws of sorted samples for X-chromosome from four different commercially available Holstein bulls that were collected and extended commercially. The sorted samples were used to test ability of method to evaluate sex ratio with high confidence.

2.4. DNA samples preparation

DNA from each semen sample was extracted by a modified protocol based on Aravindakshan et al. (1998) (see also Chandler et al., 2007). Fresh lysis solution was prepared before beginning DNA extraction. The lysis solution was made in two steps. First, 0.121 g Tris (pH = 8.0, 10 mM), 0.0293 g EDTA (pH = 8.0, 10 mM), and 80 ml distilled water were brought to pH = 8.0 using NaOH. Then, 0.584 g NaCl, 2 ml 2-mercaptoethanol and 0.5 g SDS were added. Volume was brought to 100 ml with distilled water, and the solution was stored at 2–8 °C. Samples were washed twice with 2.9% sodium citrate solution and resuspended in 50 °C lysis solution and incubated at 50 °C for 30 min. Proteinase K (Fermentas, Vilnius, Lithuania; catalog no. #EO0491) 5 μl was added to the solution and incubated at 50 °C overnight. One volume of phenol:chloroform was added and the tubes gently shaken every 3 min for 15 min at room temperature. Samples were then centrifuged at 15,500 × g for 3 min, the organic phase aspirated, and the phenol:chloroform step repeated. Two volumes of ice-cold absolute ethanol were added to the remaining aqueous phase and gently shaken to precipitate the DNA. Samples were centrifuged for 1 min
at 15,500 × g and ethanol carefully decanted. DNA pellets were dried in a vacuum until clear and resuspended in sterile water by incubating at 37 °C with gentle agitation until dissolved. Nucleic acid concentration was determined by Nanodrop (NanoDrop® ND-1000, USA).

A negative control (DNA free sample) and a positive control (DNA extracted from bull blood) were used for each assay in order to exclude, respectively, the presence of eventual contamination and to guarantee the goodness of the assay. DNA of the positive control was extracted by AccuPrep Genomic DNA Extraction Kit (Bioneer, Korea) according to the manufacturer’s instructions.

2.5. Quantitative SYBR Green Real-Time PCR

Real-Time PCR was performed on an ABI Prism 7300 Sequence Detection System (PE Applied Biosystems, USA) using Maxima™ SYBR Green qPCR Master Mix (catalog no. K0222; Fermentas, USA). DNA of each sample was run in duplicate along with a no template control (NTC) for every Assay and primed in separate wells for the two genes of interest, using a defined volumetric quantity of the same DNA sample (1 μL). Each run was completed with a melting curve analysis to confirm the specificity of amplification and lack of primer dimers.

The PCR mixture contained 1 μL of template, 0.3 μM forward primer, 0.3 μM reverse primer and 12.5 μL 2X qPCR Master Mix (qPCR Master Mix; Fermentas, USA) in ABI PRISM® 96-Well Optical Reaction Plate with Barcode (code 128). Amplifications for both genes were performed by an optimized protocol recommended by the manufacturer (10 min at 95 °C, 40 repeated cycles of two steps at 95 °C for 15 s, 58 °C for 15 s and 72 °C for 30 s).

2.6. Determination of gender chromosomes frequencies

The percentage of X- and Y-chromosome content in given semen sample (%X, %Y) was determined using the following equations (Parati et al., 2006):

\[ n = \frac{\%X}{\%Y} \text{, } \%X + \%Y = 100\% \]

2.7. Accuracy, repeatability and reproducibility assays

The accuracy of the SYBR Green method was estimated by comparing the mean value of X-chromosome frequency of a DNA sample extracted from bull blood with the expected value (μ = 50%) at four different dilutions (10^2–10^3 molecules of DNA/reaction). The repeatability (i.e., the variability of a method when repeated measures are taken with the same material in a single experiment) and reproducibility (i.e., the variability of a method when repeated measures are taken in different experiments) were assessed by computing the coefficients of variation (CV) of the X-chromosome content observed in 20 quantifications of unsorted semen samples. To this aim, distinct experiments at four different semen DNA inputs (10^5–10^6 elution of DNA/reaction) were conducted. In particular, the repeatability assay was assessed in two runs (4 replicates per run) for each sample dilution, while 20 runs (1 measure per run) were performed for the reproducibility assay.

2.8. Statistical analysis

Data from standard curves were analyzed by SDS 1.4.0.25 software (PE Applied Biosystems, USA). Threshold cycle or the Ct values of experimental samples and regression plots data were then analyzed for the interpolation analysis by the same SDS software.

A Chi-square test was used to determine if the measured X:Y semen ratio from pooled semen samples were significantly different from the expected ratio. A t-test was used to analyze data to evaluate the X-chromosome in both ejaculate and commercial semen samples. The mean values for accuracy, repeatability, reproducibility and other mentioned data were analyzed using SAS 8.2 (Institute Inc., Cary, NC).

3. Results

3.1. Sex chromosomes frequencies in pooled bovine semen samples (commercial)

The unsorted semen sample (n = 50) contained 54.7 ± 0.52% and 47.6 ± 0.60% X- and Y-sperm, respectively, which did not deviate significantly from the expected 1:1 ratio (P > 0.05).

3.2. Comparison between Real-Time PCR and sort reanalysis techniques

The estimated rates of X-bearing spermatozoa in X-sorted semen, assessed by Real-Time PCR, ranged from 91.4% to 94.2%, with an average rate of 93.3% (Table 1). The mean average for X-bearing spermatozoa in same samples that has been confirmed with flow cytometry was 98.5 (commercially sorted semen). The putative rate of Y-bearing spermatozoa assessed by Real-Time PCR ranged from 89.7% to 92.2%, with an average rate of 91.4% (Table 1). The flow cytometric result was 98.5 (commercially sorted semen). Both X/Y sex ratio in sorted semen samples were significantly deviated (P > 0.05) from the sex ratio claimed in commercial semen sorted which originated from post-sorting reanalysis data (Fig. 1A and B).

### Table 1

<table>
<thead>
<tr>
<th>Average rate of chromosome (%)</th>
<th>Average rate of chromosome (%)</th>
<th>Case number</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-sorted semen</td>
<td>Real-Time PCR results</td>
<td>93.3</td>
</tr>
<tr>
<td>Chromosome Y</td>
<td>Flow cytometry results</td>
<td>93.4</td>
</tr>
</tbody>
</table>
3.3. X-chromosome frequencies in unsorted semen samples

X-chromosome sperm frequencies were obtained in a normal distribution, ranging from 47.99% to 52.02% for commercial semen and from 49.32% to 51.89% for ejaculates (Table 2). The average and standard error values were similar in ejaculates and in commercial semen samples.

3.4. Melting curves analysis and primer specificity analysis

Analysis of melting curve illustrated there is neither primer dimer nor nonspecific products in reactions. Further analysis by agarose gel electrophoresis showed confirmed the melt curve analysis (data not shown). None of the NTC yielded any signal prior to 30 cycles, which is the upper limit for conventional PCR (Fig. 2A and B).

3.5. Standard curves

All the standard curves obtained from the validation assays showed a linear relationship \((r > 0.98)\) between the logarithm of the dilution factors and the Ct values for the serial template dilutions (Fig. 2A and B). The mean slopes of the two log–linear regression plots (X-plot and Y-plot), which represent the amplification efficiency, resulted to be similar (X: 3.01 ± 0.5; Y: 3.91 ± 0.05).

3.6. Accuracy, repeatability and reproducibility

The mean coefficients of variation (CV) resulted from the repeatability and reproducibility test were 5.59% and 5.40%, respectively. There was no significant difference \((P > 0.05)\) between the value of CV for repeatability and reproducibility assays. The mean accuracy of the method was 98.2%. The results of repeatability, reproducibility and accuracy assays at different sample dilutions \((10^5–10^2\) molecules DNA/reaction\) were shown in Table 3.

4. Discussion

A new method has been evaluated to assess bovine semen sex ratio using SYBR Green-based Real-Time quantitative PCR.

The mean sex ratio of unsorted sperm samples was found 1:1 in both ejaculates and commercial bovine semen, according to the standard meiotic model in which equivalent number of X- and Y-bearing sperms are produced. This result is consistent with other studies on bovine (Schwerin et al., 1991; Piumi et al., 2001; Parati et al., 2006). The real sexing efficiency of sorted semen was around 90% both after AI (Tubman et al., 2004; Borchersen and Peacock, 2009) and after IVF (Beyhan et al., 1999; Bermejo-Álvarez et al., 2010), which is consistent with the result of this study (93.3% X-sperm and 91.4% Y-sperm and 98.5% commercial semen sex ratio), and therefore support the method presented herein. It is obviously that sexing of IVF embryos produced by sorted semen can be considered as another method for assessing sorting accuracy that also provides information about semen fertility. There was significant difference \((P > 0.05)\) in the semen sex ratio of X/Y sorted semen as compared to the expected ratio in commercial sorted semen. These significant differences might be originated from fault report of commercial producer or might be obtained from technical fault through this study, anyway more samples analysis which flow cytometry data does not rely on commercial semen, can cause to earn a validate result.
The low mean values of CV achieved in repeatability (CV = 5.59%), reproducibility (CV = 5.40%) assays and the high rate of the mean accuracy (98.2%) demonstrated the high reliability of this new approach for quantifications of the X and Y chromosome content in semen samples, according to MIQE recommendations (Bustin et al., 2009).

The Real-Time PCR technique presented in this work revealed to be an accurate and reliable method to quantify the X- and Y-chromosome bearing spermatozoa in bovine semen. This methodology could be a valid tool, suitable for routine verification of a high number of sexed semen samples, for determination of pooled semen sex ratio or the validation and calibration of other related techniques.

**Fig. 2.** Standard curve plot for both genes (SRY and PLP genes), generated by 5 serial dilutions for every gene and melting curve analysis for SRY and PLP genes and NTC, respectively.

<table>
<thead>
<tr>
<th>No. of copies molecule DNA/reaction</th>
<th>CV for repeatability (%)</th>
<th>CV for reproducibility (%)</th>
<th>Rate of accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^5</td>
<td>5.37</td>
<td>5.17</td>
<td>97.9</td>
</tr>
<tr>
<td>10^4</td>
<td>5.49</td>
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<td>5.61</td>
<td>5.53</td>
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</tr>
<tr>
<td>10^2</td>
<td>5.90</td>
<td>5.64</td>
<td>98.7</td>
</tr>
<tr>
<td>Mean value</td>
<td>5.59</td>
<td>5.40</td>
<td>98.2</td>
</tr>
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</table>

No significant difference was found among sample dilutions in each assay (P > 0.05) and between the intra and the inter-assays (P > 0.05).

**Acknowledgments**

We would like to thank Dr. Ali Javadmanesh and Dr. Dana Thomsen for their useful comments and language editing which have greatly improved the manuscript.
thank Abbas Abad Breeding Center for providing semen samples.

References


