Determination of Sex Ratio In Bovine Semen Using SYBR Green Real-Time PCR

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Abstract

A SYBR green real-time PCR assay was developed to find out the sex skewness in bovine sex-sorted semen samples. The qPCR assay of \( PLP \) and \( SRY \) genes revealed the mean values of X- and Y-bearing spermatozoa as 50.24 ± 0.65 and 49.75 ± 0.62 per cent in unsorted, and 91.80 ± 0.79 and 8.20 ± 0.73 per cent in X-enriched semen samples respectively. The amplification efficiencies of the \( PLP \) and \( SRY \) primers were 99.25 and 98.03 per cent respectively. The method was validated by a series of repeatability and reproducibility assays which revealed low co-efficients of variations as 2.19 and 3.12 per cent respectively. Thus becoming a reliable and inexpensive tool to evaluate the sorted semen on routine basis and validation of other sperm sexing technologies.

Introduction

The main aim of a breeder is to increase the genetic potential to the maximum level and to produce a desired off-spring under optimal conditions (Pindaru et al., 2016). The scientific approach for the pre-selection of the sex of off-spring was started in 20th century (Seidel, 2012). Among several conventional techniques such as albumin gradient, swim-up, percoll density gradient, free flow electrophoresis, H-Y antigen and sorting based on volumetric differences, flow cytometry effectively sorted the X- and Y-chromosome bearing spermatozoa. This method is mainly based on the fact that X-bearing (female) spermatozoa contains 3.8 per cent more DNA than Y-bearing sperm (Johnson, 2000). Flow cytometric separation of spermatozoa was first done by Johnson et al. (1989) in rabbits. Later the technique was used successfully in several species (Johnson, 2000; Seidel and Garner, 2002; Maxwell et al., 2004; Lu et al., 2007; Liang et al., 2008).

The efficiency and success of artificial insemination with sorted semen depends on the accuracy of sexing of the spermatozoa (Pindaru et al., 2016) and high sorting accuracy is the pre-requisite for wide spread use of sexed semen (Habermann et al., 2005). The purity of sexed semen is usually verified by flow cytometric reanalysis of the DNA content of semen (Welch and Johnson, 1999). But there is a need for a reliable method, which does not depend on the same instrumentation to prevent the inherent errors of poorly sorted semen (Colley et al., 2008).

Specific DNA sequences on X- and Y-spermatozoa enable the identification of sex of individual sperm and sex ratio of semen samples (Colley et al., 2008; Maya et al., 2013). Several methods were developed to determine sperm sex ratio such as duplex PCR (Colley et al., 2008) or single cell PCR (Yadav et al., 2017), but these techniques were associated with a disadvantage of requirement of large number of individual sperms (Maleki et al., 2013; Yadav et al., 2017). Quantitative real-time PCR (qPCR) technique emerged subsequently with some advanced features for the sex determination of spermatozoa more simply and accurately in pooled semen samples (Maleki et al., 2013). Quantitative PCR allows simultaneous amplification of the DNA and real time monitoring of amount of amplified DNA through reaction cycles. Therefore, the present study was aimed at development and validation of SYBR green real-time PCR assay for the quantification of X and Y-bearing spermatozoa in bovine semen samples.
Materials And Methods

Semen samples and sperm DNA extraction

A total of seven unsorted and four X-enriched frozen semen samples were thawed and washed twice with phosphate buffered saline. The sperm pellet was resuspended in 2.5 ml of sperm lysis buffer (50 µl of 1M Tris-HCl, 100 µl of 0.5M EDTA, 100 µl of 5M NaCl, 0.1 g of 4% SDS and 50 µl of 2% Mercaptoethanol) and 2.2 ml of nuclease free water by vortexing and incubated for 20 minutes at 56 °C in a water bath. To the suspension, DNAzol (Thermo Scientic, USA) was added, mixed thoroughly and incubated for 2-3 minutes at room temperature. Later, Phenol:Chloroform:Isoamylalcohol (25:24:1) was added, mixed well by vortexing and centrifuged at 11,000 rpm for 10 minutes. The aqueous phase was collected into a separate 2ml eppendorf tube, added 24:1 ratio of chloroform:isoamylalcohol and mixed throughly by vortexing. After centrifuging at 11,000 rpm for 10 minutes, aqueous phase was collected into a separate tube, equal volume of isopropanol added, centrifuged and the supernatant discarded. Later, the DNA pellet was treated with 70 per cent and absolute alcohol, air-dried and resuspended in 50 µl of TE buffer. The purity and concentration of DNA samples were estimated by gel electrophoresis and spectrophotometry (NanoDrop OneC of Thermo Scientic, USA).

Primers

Gene specific nucleotide sequence was obtained from NCBI for PLP gene (Genbank Accession No. AJ009913.1) and cattle X-specific PLP primers were designed using Primer-BLAST software. The forward and reverse primer sequences for PLP gene were 3'-ACC AAG GGA AGA GCA GGA AT-5' and 3'-TGC CAA CTT GTA CCC AAA CA-5' respectively. The primers for Y-specific SRY (Sex determining region Y) gene was synthesized as described by Hamilton et al. (2009). PCR amplification was carried out using thermal cycler (Applied Biosystems 2720).

Sybr Green Real-time Assay

The quantitative PCR was performed with 5 µl of SYBR green mix (Sso A dvanced Universal SYBR Green super mix, Bio-Rad), 3 pmol each of forward primer and reverse primers (0.3 µl), 70 ng of sperm DNA as template (1 µl) and the final volume of reaction was made up to 10 µl by nuclease free water. The amplification profile consisted of initial denaturation at 95°C for 2 minutes, denaturation at 94 °C for 30 seconds, annealing at 64°C for 30 seconds, extension at 72°C for 30 seconds and melt curve at 72-98°C with every 0.2°C hold for 1 second. The amplified PCR products were checked on 2 per cent agarose gel with 100 bp DNA ladder. The bands were viewed in a GelDoc (Bio-Rad Laboratories Inc., USA) system and the images stored.

Generation Of Standard Curve
PLP and SRY genes were amplified in a 50 µl PCR reaction mixture. PCR products were resolved with 2 per cent agarose gel and bands were sliced under UV illumination. The bands were purified from gel with Favrogen gel/PCR purification kit (Favorgen, Taiwan). The concentration of purified PCR products was measured using NanoDrop. The specificity of the PCR product was rechecked with 2% agarose gel. Using concentration of DNA and product length, copy number per µl volume was calculated with online calculator for determining the number of copies of a template (URI Genomics and Sequencing Centre). The concentration of the stock solution was adjusted to 1 X 10⁶ copies. Then, the stock solution was diluted serially by 10 folds to obtain a standard series from 1 X 10⁶ to 1 X 10² copies. A standard curve was constructed with different copy numbers in triplicates. The Cₜ values obtained during amplification of each dilution were plotted against logarithm of their template dilution factor. The coefficient of determination (R²) value obtained from the equation of linear regression line was used to evaluate the qPCR assay.

Validation of quantitative PCR assay

Repeatability (intra-assay) means the variability of a method when repeated measures are taken with the same material in a single experiment, while reproducibility (inter-assay) is the variability of a method when repeated measures are taken in different experiments (Maleki et al., 2013). Repeatability was calculated by computing the co-efficients of variation (CV) of X-chromosome (PLP gene) content observed in 20 quantifications of the same unsexed semen sample. Hence, repeatability of the experiment was calculated by conducting 10 repetitions for each dilution (10⁸ to 10²) in two runs of PLP gene. The co-efficients of variation for reproducibility was calculated by performing one measure per run for each dilution (10⁸ to 10²) in 10 runs of PLP gene.

Calculation of copy numbers and percentage of X- and Y-spermatozoa

Copy numbers of unknown samples were calculated by using the formula (Real-Time PCR Application Guide, Bio-Rad)

\[ Cn = 10^{(ct-b)/m} \]

Where, Cn= copy numbers; Ct = Ct value of unknown sample; b = intercept of standard curve; m = slope of the standard curve.

Copy numbers for each gene was calculated separately and converted into percentages by using the formula suggested by Parati et al. (2006). A Chi-square test was used to determine whether the observed percentages of X- and Y-spermatozoa in a semen samples differed significantly from expected sex ratio 1:1. The data were analyzed with IBM SPSS Statistics 20 software.

Results
Primer specificity analysis

The primers used for PLP and SRY genes showed uniqueness in amplifying a single PCR product in real time assay as there was neither primer dimer nor non-specific products in reaction. Further analysis by agarose gel confirmed the melt curve analysis. None of the control yielded any signal prior to 30 cycles. Both the primers showed a single melting peak at temperatures 82.20 °C (PLP) and 84.40 °C (SRY) as shown in Fig. 1.

Standard curve

The standard curves obtained showed a linear relationship (R²=0.99) between logarithm of dilution factors and Ct values for serial template dilutions (Fig. 2). The Ct values ranged from 8.55 (1x10^8) to 27.34 (1x10^2) for PLP gene and 10.38 (1x10^8) to 28.59 (1x10^2) for SRY gene (Table 1). The end point of qPCR revealed a clear distinct band in decreasing intensity as a result of serial dilution of the amplicons without formation of any multiple non-specific amplification (Fig. 3).

Table 1. Ct values of standard curves for PLP and SRY genes for serial template dilutions using quantitative real-time PCR

| Sl. No. | No. of copies | Ct values |
|---------|---------------|-----------|
|         |               | PLP       | SRY       |
| 1       | 1x10^2        | 27.34     | 28.59     |
| 2       | 1x10^3        | 25.18     | 26.69     |
| 3       | 1x10^4        | 22.26     | 23.04     |
| 4       | 1x10^5        | 19.23     | 20.32     |
| 5       | 1x10^6        | 15.41     | 16.6      |
| 6       | 1x10^7        | 12.22     | 13.03     |
| 7       | 1x10^8        | 8.55      | 10.38     |

The general linear equations (y=mx+b) obtained for both the genes were

y = -3.183x + 31.33 for PLP gene

y = -3.156x + 32.43 for SRY gene

Figure 2. Standard curve of (a) PLP and (b) SRY genes
Repeatability And Reproducibility Assay

The mean co-efficient of variations resulted from the repeatability and reproducibility assays were 1.45 and 2.69 respectively (Table 2). No significant difference was found among sample dilutions in repeatability and reproducibility assays (P>0.05)

Absolute quantification of allosome frequencies

The mean percentage of X- and Y-bearing spermatozoa in unsorted semen samples were 50.24 ± 0.65 and 49.75 ± 0.62 respectively. The range of X- and Y-chromosome bearing spermatozoa varied from 45.94 to 56.70 and 43.29 to 54.05 per cent respectively (Table 3).

Table 2. Co-efficients of variation (CV) for the assays of repeatability and reproducibility at different dilutions of unsorted DNA molecules

| No. of copies of DNA molecules/reaction | CV for repeatability (%) | CV for reproducibility (%) |
|----------------------------------------|--------------------------|----------------------------|
| 1×10⁸                                  | 4.48                     | 5.7                        |
| 1×10⁷                                  | 1.73                     | 2.2                        |
| 1×10⁶                                  | 1.95                     | 2.03                       |
| 1×10⁵                                  | 2.76                     | 4.23                       |
| 1×10⁴                                  | 1.79                     | 3.89                       |
| 1×10³                                  | 1.4                      | 2.31                       |
| 1×10²                                  | 1.27                     | 1.5                        |
| Mean                                   | 2.19                     | 3.12                       |

The mean values for X- and Y-bearing spermatozoa in X-enriched semen samples were 91.80 ± 0.79 and 8.20 ± 0.73 per cent, ranging from 89.72 to 94.37 and 5.63 to 10.28 respectively (Table 3). While the Chi-square statistical test of goodness of fit revealed a significant difference (P<0.01) between the X- and Y-bearing spermatozoa in the X-enriched semen samples and no significant difference between the observed and expected per cent of X- and Y-spermatozoa in unsorted semen samples with a mean sex ratio equal to 1:1 (50.24 : 49.75) across all the tested unsorted semen samples.

Discussion
Real-time quantitative PCR for determination of sperm sex ratio carried out in this study gave an accurate result. PLP and SRY genes were used as gender markers for identification of X- and Y-bearing spermatozoa respectively. Both the genes are present in single copy on X- and Y-chromosomes, therefore, every single copy of PLP and SRY sequence detected indicated the presence of respective quantities of X- and Y-bearing spermatozoa (Tan et al., 2015).

Since all gene sequences for a given species are represented, genomic DNA could serve as universal standard for the absolute quantification of any expressed gene (Yan et al., 2006). In this study, purified PCR products of PLP and SRY genes were used to generate the standard curves. Both the genes gave different Ct values with same gene copy numbers due to differences in the length of gene fragments (Tan et al., 2015). Therefore, two different standard curves were obtained for quantification of X- and Y-chromatin bearing spermatozoa. Lower the mean values of co-efficient of variation for repeatability and reproducibility, higher would be reliability of this approach in absolute quantification of X- and Y-chromatin contents in semen samples.

From the copy numbers of PLP and SRY genes, the quantity of X- (50.24%) and Y- (49.75%) chromosome bearing spermatozoa did not differ from 1:1 ratio, which is reported as normal in unsexed mammalian semen samples (Rosenfeld and Roberts, 2004; Luna Estrada et al., 2006; Whyte et al., 2007). According to the standard meiotic model, equal proportion of X- and Y-bearing spermatozoa are produced in semen (Maleki et al., 2013) and this advantage was utilized to optimize the technique. The sex ratio obtained in unsorted semen samples of this study was also supported by previous reports made by different experiments in sex determination (Piumi et al., 2001, Rens et al., 2001, Revay et al., 2002, Habermann et al., 2005, Parati et al., 2006, Colley et al., 2008, Maleki et al., 2013, Tan et al., 2015; Reinsalu et al., 2019).
| Type of sample | X-bearing spermatozoa | Y-bearing spermatozoa |
|---------------|-----------------------|-----------------------|
|               | Mean ± S.E. | Range  | Mean ± S.E. | Range  |
| Unsorted semen |           |        |           |        |
| 1             | 50.74 ± 2.52 | 47.44-54.21 | 49.24 ± 2.52 | 45.79-52.55 |
| 2             | 49.03 ± 3.23 | 46.74-51.31 | 50.97 ± 3.23 | 48.68-53.25 |
| 3             | 49.41 ± 4.23 | 45.94-54.11 | 50.59 ± 4.22 | 45.88-54.05 |
| 4             | 49.76 ± 0.62 | 49.31-50.20 | 50.24 ± 0.622 | 49.79-50.68 |
| 5             | 50.96 ± 2.94 | 48.87-53.04 | 49.04 ± 2.94 | 46.95-51.12 |
| 6             | 53.54 ± 4.47 | 50.37-56.70 | 46.45 ± 4.47 | 43.29-49.62 |
| 7             | 48.27 ± 2.95 | 46.18-50.36 | 51.73 ± 2.95 | 49.63-53.81 |
| Overall       | 50.24 ± 0.65 | 45.94 -56.70 | 49.75 ± 0.62 | 43.29 - 54.05 |
| X-enriched semen |         |        |           |        |
| 1             | 93.53 ± 0.54 | 92.52-94.37 | 6.47 ± 0.54 | 5.63-7.48 |
| 2             | 92.73 ± 0.73 | 91.29-93.66 | 7.27 ± 0.73 | 6.34-7.48 |
| 3             | 90.79 ± 0.52 | 89.86-91.69 | 9.21 ± 0.52 | 8.31-10.14 |
| 4             | 90.14 ± 0.26 | 89.72-90.63 | 9.86 ± 0.26 | 9.37-10.28 |
| Overall       | 91.80 ± 0.79 | 89.72 - 94.37 | 8.20 ± 0.73 | 5.63 - 10.28 |

To conclude, the sex determination in bovine unsorted semen samples was performed successfully with SYBR Green fluorescent dye. The SYBR Green real time quantitative PCR is a rapid and reliable technique in absolute quantification of sex ratio in bovine semen samples. This method is a valid tool for routine validation of high number of sorted semen samples, determination of sex ratio in pooled semen samples and can also be used for the validation or calibration of other related techniques.

**Statements And Declarations**

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Availability of data and material: The data set generated and analyzed during the current study are not publicly available due to confidential reasons, but are available from the corresponding author on reasonable request.

Ethical statement: The manuscript does not contain clinical studies or patient data

Consent to participate: Not applicable

Consent for publication: Not applicable

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Figures

**Figure 1**

Melting curve of (a) PLP gene (82.20ºC) and (b) SRY gene (84.40 ºC)

**Figure 2**
Standard curve of (a) PLP and (b) SRY genes

Figure 3

Gel electrophoresis (2% agarose) revealing gradual reduction in the intensity of amplicons upon serial dilutions for PLP and SRY genes (Lanes 1-7: PLP gene dilutions from $1 \times 10^8$ to $1 \times 10^2$; Lane 8: 50 bp marker; Lanes 9-15: SRY gene dilutions from $1 \times 10^8$ to $1 \times 10^2$)