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Development of qPCR Assay for Determination of Sperm Sex Ratio in Indian Cow Bull

Richa Khirbat[1], Aman Kumar[1], Trilok Nanda[1], Sushila Maan[1]

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ABSTRACT

Background: Currently, the sex selection of cattle offspring holds great promise for genetic advancement and meeting consumer demand. Sperm sorting by flow cytometry provides a reliable tool for artificial insemination and the creation of embryos with predetermined sexes. A precise evaluation of the yield of sperm separation is still needed for a field application of this method or for the advancement and validation of other related semen sexing technologies. The current study was conducted to develop a qPCR-based method for the determination of sperm sex ratio in cow bull semen.

Methods: The SYBR Green-based Real-Time PCR chemistry targeting *PLP* (X chromosome-specific gene) and *SRY* (Y chromosome-specific gene) genes were used for the development of the assay. To determine the efficiency of PCR amplification, standard curves were generated.

Result: The developed assay revealed a negligible variation in the semen sex ratio (51.7±0.465% X and 48.23±0.465% Y) of unsorted semen. The repeatability and reproducibility of this approach were evaluated. For *PLP* and *SRY*, the standards produced a linear relationship with regression coefficients of 0.994 and 0.997, respectively. The low mean values of CV obtained in repeatability and reproducibility trials demonstrated the high dependability of this novel method for assessing the sexual chromosomal content in semen samples.

Key words: Enrichment, Sperm sex ratio determination, SYBR green real-time PCR.

INTRODUCTION

The ability to regulate progeny's sex is most important for the goal-oriented development of the livestock sector (Garner and Seidel, 2008; Karabinus, 2009). Since, it provides economically flexible management approaches for animal producers, sorting X- and Y-spermatozoa prior to conception is an obvious method to accomplish the task (Rath *et al*., 2008). Recently, assisted reproductive technologies (ART) like artificial insemination (AI), *in vitro* fertilization (IVF) and embryo transfer (ET) implies direct applications of sperm sexing technology (Parati *et al*., 2006). Various approaches like percoll density gradient, albumin gradient, swim-up, free flow electrophoresis and H-Y antigen-based sorting were attempted by researchers to separate X and Y spermatozoa. At present, flow cytometry is the only quantitative and reasonably accurate method for sexing mammalian sperm, which is based on difference in the amount of DNA in X and Y spermatozoa. However, the difference in the amount of DNA between X and Y spermatozoa varies from 3.8%-4.98% depending on cattle breeds (Kawarasaki *et al*., 1998; Johnson and Welch 1999; Prakash *et al*., 2014). Even though FACS provides a trustworthy tool for artificial insemination and the production of embryos with predetermined sexes, a precise assessment of the yield of sperm separation is still required for a field application of this technique.

Determining the ratio of X and Y spermatozoa in semen is of great interest to evaluate the efficiency of semen sorting procedure (Maleki *et al*., 2013). Usually, the sort reanalysis is the convenient and most accurate method of evaluating the sorted spermatozoa wherein the sorted sperms are reanalyzed using same flow cytometry instrument. The use of same instrumentation for sort reanalysis carries over the inherent errors resulting in poor quality of sorted semen (Colley *et al*., 2008) and large number of spermatozoa are required for analysis (5×10^4 sperm) (Welch *et al*., 1999). At the same time deviation in ratio of male and female offspring in the population from expected 1:1 arouse interest in this kind of evaluation (Clutton-Brock *et al*., 1986). Multicolour FISH (fluorescent in situ hybridization) is the most reliable method for single sperm sexing using sex specific probes resulting in hybridization signals (Rens *et al*., 2001; Piumi *et al*., 2001; Di Berardino *et al*., 2004; Habermann *et al*., 2005).

The differences in the sequence of DNA of X and Y chromosome were exploited for evaluation of sex ratio of

[1]Department of Animal Biotechnology, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar-125 004, Haryana, India.

Corresponding Author: Aman Kumar, Department of Animal Biotechnology, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar-125 004, Haryana, India. Email: aman.abt@luvas.edu.in

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semen samples (Colley *et al*., 2008). Real-time PCR assay is one of the reliable methods to determine the ratio of X and Y spermatozoa in semen sample. Initially, TaqMan probes specific for X and Y chromosome *i.e.* for proteolipid gene (*PLP*) and male sex determination gene (*SRY*) respectively were used by Joerg *et al.* (2004) and Parati *et al.* (2006). Quantitative Real time PCR allows more accurate and simple method of sex determination of spermatozoa. The objective of the present study is to develop and validate SYBR green Real time assay for sex determination of spermatozoa in cow bull (*Bos indicus*) semen.

MATERIALS AND METHODS

DNA extraction

Four samples each from unsorted (frozen) semen of four different bulls of Hariana breed, cow bull blood and enriched semen were used for the study. The DNA isolation was performed using Quick-DNA™ MiniprepPlus Kit (Zymo Research, Cat no. -D3024) protocol with minor modifications. Briefly, 200 µl semen sample aliquot was mixed with 200 µl of Bio fluid and cell buffer with the subsequent addition of 20 µl proteinase K and 20 µl 1M DTT (DiThioThritol) (SRL). The mixture was vortexed for 30 seconds incubated at 56°C for 2 hr. After incubation the mixture was transferred to a Zymo-Spin™ IIC-XLR Column with collection tube and centrifuged at ≥ 12,000 × g for 1 minute. Flow through was discarded and washing was done by using 400 µl of DNA pre wash buffer at 12,000 × g for 1 minute. Further two more washings were done with 700 µl of g-DNA Wash Buffer and 200 µl of g-DNA Wash Buffer respectively at 12,000 × g for 1 minute. Finally, elute was taken in 50 µl of elution buffer and stored in a new tube at -20°C for further use. Further, DNA quality and quantity were measured by using nanodrop (Smart specTM Plus, BIO-RAID). All the sampling was done in accordance with guideline and regulations of Institutional Animal Ethics Committee (IAEC), LUVAS, Hisar.

Primers

A primer pair specific to X and Y chromosome in cattle (*Bos indicus*) were used for SYBR green real-time PCR in accordance with the parameters required for SYBR green Real time PCR (Dorak, 2006). The Y-specific primer pair for *SRY* gene has been taken from previously published paper of Kumari *et al*., (2019) with amplification product length of 142 bp. However, the X- specific primer pair was designed on a conserved region of the *Bos taurus* proteolipid protein gene (*PLP*) (GenBank accession NM_174149.4) using IDT oligocalculator tools (https://sg.idtdna.com/pages/tools/oligoanalyzer) with amplification size of 110 bp. The details of forward and reverse primer of *PLP* and *SRY* gene are given in Table 1.

Quantitative SYBR green Real-Time PCR

Real-Time PCR was performed on anCFX Opus Real-Time PCR Systems (BIORAD)using VeriQuest™ SYBR™ Green qPCR Master Mix (2X) (Cat. no. 75600; Thermo Fischer Scientific). The quantitative PCR was performed with 5 µl of SYBR green mix, 0.3 µl each of forward primer and reverse primers (10 pmol), 2 µl sperm DNA as template and the final volume of reaction was made up to 10 µl by nuclease free water. Using a predetermined volumetric amount of the same DNA sample, each sample's DNA was run in duplicate along with an NTC for each assay and primed in separate wells for the two genes of interest. A melting curve analysis was performed at the end of each run to ensure the specificity of the amplification and the absence of primer dimmers. Amplifications for both genes were performed by an optimized protocol (2 min at 95°C, 39 repeated cycles of three steps at 95°C for 5 s, 60°C for 30 s and 72°C for 30 s and melt curve at 95°C for 5 s, 65°C for 5 s and 95°C for 50 s). The amplified PCR products were checked on 2.5% 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.

Establishment of standard curve

A dilution series of known template concentrations was used to establish a standard curve for determining the amount of the target template *i.e*. X and Y bearing spermatozoa as well as assessing the reaction efficiency. The concentration of the stock template DNA (cow bull blood) was checked using nanodrop(Smart specTM Plus, BIO-RAID). Then, the10-fold serial dilutions of stock solution were used to obtain a standard curve from 1×10^{-1} to 1×10^{-5} dilution. To create a standard curve, various dilutions were used in duplicate. The Ct values obtained during amplification of each dilution were plotted against logarithm of their template dilution factor. The qPCR assay was evaluated using the coefficient of determination (R^2) value that was derived from the equation of the linear regression line.

Determination of gender chromosome frequency

The following equations were used to calculate the percentage of X chromosomes in a given semen sample:

$$\%X = (n/n+1)100,\ \%X + \%Y = 100$$

Where:

$$n = \frac{CO_X}{CO_Y}$$

n= Ratio of proportion of X and Y in the semen sample.

Which corresponds to the relative amount of X and Y (expressed as dilution) observed on each standard linear plot and referenced to the respective mean ct values (Parati *et al*., 2006; Maleki *et al*., 2013).

Repeatability and reproducibility assays

Reproducibility indicates the variability of the method when repeated measures are taken in various experiments, while repeatability measures the variability of the assay when repeated parameters are taken using the same material in a single experiment. To this aim, repeatability was calculated

by computing coefficient of variation (CV) of X chromosome (*PLP* gene) content was observed in four quantifications for each dilution (10^{-1} to 10^{-5}) in duplicate. The coefficient of variation for reproducibility was calculated by performing one measure per run for each dilution (10^{-1} to 10^{-5}) in four runs of *PLP* gene. 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.

Table 1: Details of primers used in SYBR green Real-time PCR.

Primer pair	Primer size (bp)	Primer sequence (5′-3′)	Amplicon size
PLP F	22	CAGGCAGATCTTTGGCGACTAC	110 bp
PLP R	22	CTTGATGTTGGCCTCTGGAACC	
SRY F	20	ACGCCTTCATTTTGTGGTCT	142 bp
SRY R	20	TCCTCAAAGAATGGGCGCTT	

Table 2: Percentage of X and Y chromosome bearing spermatozoa in unsorted semen, cow bull blood and X enriched semen samples.

Types of samples	X-bearing spermatozoa (%)	Y-bearing spermatozoa (%)
	Mean±S.E.	Mean±S.E.
Unsorted semen	51.9±0.70	48.1±0.70
	51.9±0.93	48.1±0.93
	53.16±0.11	46.84±0.11
	50.12±0.12	49.88±0.12
Male blood	51.21±0.0	48.79±0.0
	51.91±0.47	48.09±0.47
	49.87±0.13	50.13±0.13
	50.97±0.24	49.03±0.24
X-enriched semen	81.23±1.64	18.77±1.64
	79.95±0.32	20.05±0.32
	79.07±0.41	20.93±0.41
	75.67±2.63	24.33±2.63

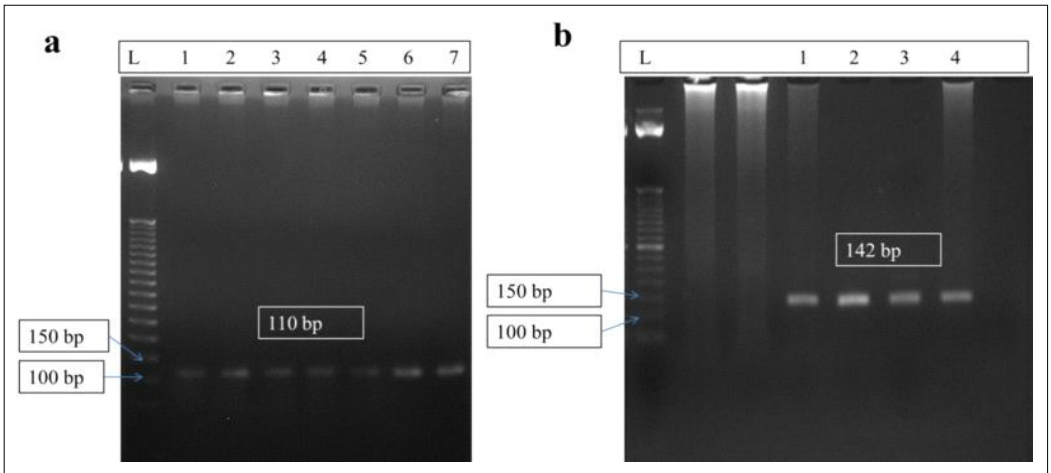
RESULTS AND DISCUSSION

DNA extraction

DNA was isolated from unsorted (frozen) semen and cow bull blood (Hariana) Quick-DNA™ MiniprepPlus Kit (Zymo Research, Cat no. -D3024) protocol with minor modifications. Further, DNA quality and quantity were measured by using nanodrop. The quality of extracted DNA was determined using the ratio of absorbance at 260 and 280 nm. For the nanodrop, 1 µl of the undiluted sample was used to read the absorbance and the elution buffer of the kit was used as blank. The average DNA concentration was 47.86 ng/µl with the absorbance at 260/280 ratio lying between 1.8-1.9 which falls in the acceptable range for subsequent applications. Isolation of intact, highly concentrated, uncontaminated genomic DNA is a prerequisite for the success of PCR-based molecular methods (Grom *et al*., 2006; Manuja *et al*., 2010; Sharifzadeh *et al*., 2011). Unlike somatic cells, sperm DNA is very compact due to the replacement of histones with protamines and disulfide bridges formed within and between the protamines (Griffin, 2013). The unique DNA packaging renders spermatozoa resistant to DNA isolation techniques used for somatic cells. Therefore, modifications *i.e*. addition of 0.1 M DTT and incubation at 56°C for 2 hours after the addition of proteinase K in combination with Quick-DNA™ MiniprepPlus Kit (Zymo Research) extraction protocol gave better results because DTT is basically a reducing agent which acts on the outer membrane and chromatin of spermatozoa which contains disulfide bonds.

Determination of allosome frequencies

The mean percentage of X and Y-bearing spermatozoa in unsorted semen samples were 51.7±0.465 and 48.23±0.465 respectively. The mean percentage of X and Y-bearing spermatozoa in cow bull blood were 50.9±0.21 and 49.01±0.21 respectively (Table 2). The mean percentage of X and Y-bearing spermatozoa in the X-enriched sample were 78.85±1.25 and 21.14±1.25 respectively (Khirbat., 2022).



(a); L-50 bp ladder; Lanes 1-7 -*PLP* gene amplicons (110 bp) (b); L-50 bp ladder Lanes 1-4 -*SRY* gene amplicons (142 bp).

Fig 1: Agarose Gel Electrophoresis of Real time PCR amplicons of *SRY* and *PLP* gene on 2.5% gel.

With a mean ratio of 1:1 across all the samples tested, the chi-square test of goodness of fit shows that there is no significant difference between the observed and predicted (1:1) percentage of X and Y spermatozoa in unsorted semen samples and cow bull blood. This result is consistent with similar studies conducted on bovine (Parati *et al*. 2006; Colley *et al*. 2008; Maleki *et al*., 2013; Tan *et al*., 2015). Additionally, the mean percentage of X and Y spermatozoa in the X-enriched samples showed a significant difference ($p<0.05$) in the chi square test. PLP and SRY genes were utilized as markers in this assay to distinguish between spermatozoa containing X and Y, respectively. Tan *et al.* (2015) claim that because both the PLP and SRY genes are present on the X and Y chromosomes in a single copy, the number of copies that can be found can be used to estimate the number of spermatozoa that carry the X and Y chromosomes. The inclusion of unsorted semen in the study offers an added benefit for optimising the qPCR reactions since it contains the known fraction of spermatozoa bearing the X and Y chromosome.

Table 3: Ct values of standard curve for PLP and SRY gene serial template dilutions using quantitative real time PCR.

Log dilutions	Ct values	
	PLP	*SRY*
1	20.75	18.7
1×10^{-1}	25.3	22.96
1×10^{-2}	29.07	26.12
1×10^{-3}	32.67	29.5
1×10^{-4}	35.6	32.85
1×10^{-5}	38.9	35.33

Primer specificity and melting curve analysis

Melting curve analysis shows that primers used for *PLP* and *SRY* genes were amplifying a single PCR product with neither primer dimer and nor nonspecific products. Subsequently melt curve analysis was confirmed by agarose gel electrophoresis (Fig 1). No signal was observed in any NTC before 30 cycles. Both the primers showed a single melting peak at temperatures 81.0°C (*PLP*) and 77.5°C (*SRY*) as shown in Fig 2. When singleplex PCR is performed, real-time PCR analysis based on fluorescent DNA dyes, such as SYBR-Green, has a number of advantages over sequence-specific probes. Compared to other dyes that are currently known, this dye is the simplest and least expensive. As a result of SYBR Green's propensity to bind to all double-stranded nucleic acid molecules, it can be used to detect the build-up of primer dimers and the amplification of non-specific PCR products (Deprez *et al*., 2002).

Standard curve

The standard curve obtained in the assay showed linear relationship ($r^2> .99$) between logarithm of dilution and Ct values for serial template dilution (Table 3; Fig 3a and 3b). The general linear equation for both the genes were:

y = -3.28x +38.84 (*SRY* gene).

y= -3.58x+ 42.91 (*PLP* gene).

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: 1.90; Y: 2.01) *i.e.* 90.25% and 101.74% respectively.

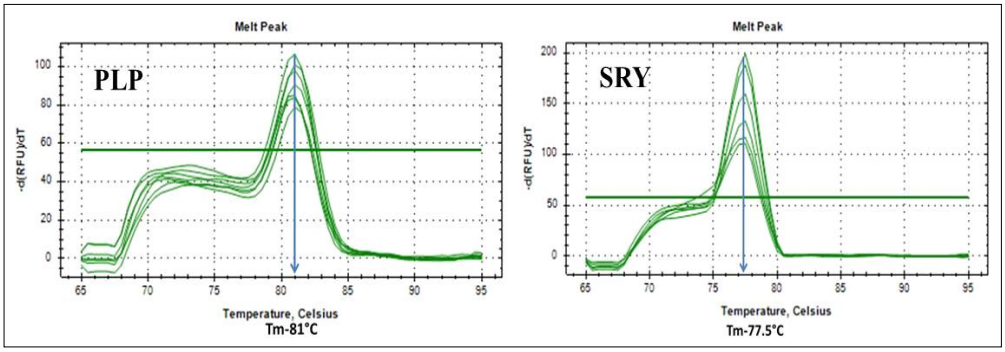


Fig 2: Melt curve of *PLP* and *SRY* genes.

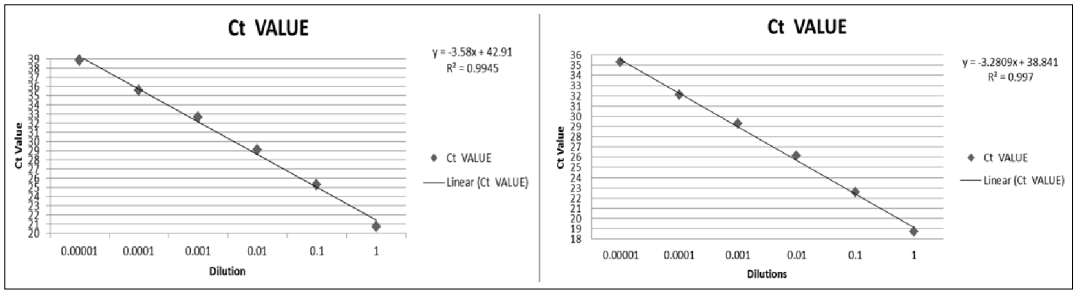


Fig 3: (a): Standard curve of *PLP* gene, (b): Standard curve of *SRY.*

Table 4: Coefficient of variation (CV) for the assays of repeatability and reproducibility at different dilutions.

Dilution	CV for repeatability (%)	CV for reproducibility (%)
10^{-5}	1.99	0.57
10^{-4}	2.12	1.33
10^{-3}	0.96	0.10
10^{-2}	2.07	0.92
10^{-1}	0.60	0.84
1	5.31	2.39
Mean	2.17	1.025

Repeatability and reproducibility

The repeatability and reproducibility tests yielded mean coefficients of variation (CV) of 2.17 and 1.025, respectively. The CV values for the repeatability and reproducibility tests did not differ significantly (P>0.05). Table 4 displays the results of tests for repeatability and reproducibility at different sample dilutions (10^{-5}-10^{-1}) DNA molecules/reaction. In accordance with MIQE recommendations, the low mean values of CV obtained in the repeatability (CV = 2.17%) and reproducibility (CV = 1.025%) experiments demonstrated the great dependability of this novel approach for quantifications of the X and Y chromosomal content in semen samples (Bustin *et al*., 2009). The X- and Y-chromosome-bearing spermatozoa in bovine semen can be quantified using the Real-Time PCR approach that was provided in this study. This process may be a reliable tool for routinely confirming many sexed semen samples, calculating the sex ratio of pooled semen, or validating and calibrating other related techniques.

CONCLUSION

The SYBR green chemistry-based Real-Time PCR has been developed successfully with limit of detection 5.123pg/µl of sperm DNA. The developed assay determines the concentration of X- and Y-chromosome-bearing spermatozoa of Indian cow bull very efficiently.

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Conflict of interest

There is no conflict of interest among the authors.

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