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Verification of Caprine Sexed-separated Spermatozoa by Real Time PCR

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Abstract

Sexed spermatozoa could contribute to increasing profitability of the dairy and beef industries worldwide by producing offspring of predetermined sex. Various methods have been designed to separate sperm population into X- and Y-chromosome bearing spermatozoa. In this study, the caprine semen sample from Savanna (n=2) and Boer (n=4) breeds were sex-separated using free flow electrophoresis. In order to determine the efficiency of sex-separation procedure, a rapid and accurate technique real time PCR (qPCR) was used to validate the X- and Y-chromosome bearing spermatozoa following separation. The primers and probes were developed to detect the X- and Y- chromosome bearing spermatozoa of *Capra hircus* based on ZFX and SRY genes, respectively, using TaqMan qPCR assay. The amplification of ZFX and SRY genes produced a single fragment of 93 bp and 137 bp, respectively. The primers used in separated spermatozoa by free-flow electrophoresis technique showed that the mean ratio of X- and Y-chromosomes bearing spermatozoa from the cathodic and anodic fractions were 36:64 and 44:56, respectively, compared to unseparated semen was 51:49. Thus, these primers can be used to validate the gene copy of X- and Y-chromosome bearing spermatozoa in caprine semen.

Key words: sperm sexing, free-flow electrophoresis, X- and Y- bearing chromosome, Real time PCR, caprine

Introduction

In the animal industry, predetermination of sex of offspring gives an important role in the economic and management systems (Hamano, 2007). Several advantages have been suggested for producing sexed spermatozoa in cattle, such as the opportunity of using fewer and genetically better cows for producing replacement dairy heifers (Prasad *et al*., 2010), a wider chance for crossbreeding of dairy cows and less number of cows required for progeny testing (Hohenboken, 1999). Moreover, sperm sexing will also facilitate the conservation of endangered species (Garner, 2006). The production of sexed spermatozoa, either X- or Y- chromosome, depends much on the economics and efficiency of use. The females are important for the production of milk and calves and lambs, whilst males are desired for beef production and artificial insemination programs (Prasad *et al*., 2010; Sureka *et al*., 2013).

Sexing of spermatozoa in goats is not a priority compared to cattle. In the cattle industry, the production of sexed semen is being used commercially (Jain *et al*., 2011). However, to date, the demand for goat milk and downstream products is rising as a result

of demographic and social changes in society (Mace, 2009). In the meantime, efforts have been made to separate the caprine spermatozoa into X- and Y-chromosome to increase the profit in livestock industry. In dairy goat production, artificial insemination has been adopted using non-sexed fresh and frozen-thawed sperm but not for sexed semen. Various procedures are available to separate spermatozoa into X- and Y- chromosome bearing spermatozoa. The techniques were developed based on the differences between each subpopulation on the basis of mass, motility, DNA content, surface charge and sperm surface antigenic determinants (Prasad *et al.*, 2010). The methods of fractionation such as H-Y antigen, sex-specific antibody binding, density gradient antibody, electrophoresis, albumin centrifugation, sedimentation, sephadex gel and flow fractionation were also used for the separation of semen sample (Sureka *et al*., 2013). The free-flow electrophoresis method was revealed to be able to separate spermatozoa into X- and Y-chromosome bearing spermatozoa through a charge from an anode or a cathode (Kaneko *et al*., 1984 and Engelmann *et al*., 1988). This procedure is an inexpensive, fast and capable method for gamete separation (Engelmann *et al*., 1988). However, a more reliable and rapid technique is needed to verify the proportion of X- and Y-chromosome following separation.

Many techniques have been used to evaluate the true chromosomal content of sperm cells after sex-separation procedures including Fluorescence *in situ* Hybridization (FISH) (Somarny *et al*., 2011; Yan *et al*., 2006), quinacrine mustard staining for Y-chromosome (Blottner *et al*., 2009) and southern blotting. Recently, the most effective, accurate and rapid technique for validation of gene copy number is relied on the PCR-based method which requires a small quantity of genomic DNA. The real time PCR, qPCR is the best choice of validation techniques for gene copy number compared to the conventional PCR where it requires quantification. Parati *et al*. (2006) had successfully developed TaqMan probes for the determination of X- and Y-chromosome bearing spermatozoa in bovine semen based on PLP and SRY genes copy number, respectively. A study by Maleki *et al*. (2013) also performed SYBR green real time PCR to determine the sex ratio in bovine. Thus, the present study was carried out to develop primers and probes for the quantification of X- and Y-chromosome bearing spermatozoa in the caprine semen samples using TaqMan Real-Time PCR assay.

Materials and Methods

Semen Preparation and Sex-separation Procedure

Semen samples were collected twice a week via artificial vagina from six fertile bucks (aged 18 - 36 months) of Savanna and Boer breeds and evaluated immediately under the microscope to determine the quality of samples including wave of semen, motility and concentration of spermatozoa per ejaculation using sperm analyzer (IVOS, Hamilton Thorne). The semen sample was selected based on quality standards of wave 4-5, motility > 70% and total spermatozoa per ejaculate > 1 X 10^9 before proceeding to sex-separation procedure. The clean chamber was filled with BioXcell buffer (IMV Technologies, France) and equilibrated for 30 min before introducing the semen sample. Approximately 300 µL of fresh semen at a concentration of 1 × 10^9 spermatozoa/mL was layered into the middle of chamber containing buffer under electric field to separate X- and Y-chromosome bearing spermatozoa at 24 to 26 ^oC for 1.5 h. The samples were run at constant applied current

of 3 V. At the end of the process, the separated semen sample was collected to assess for motility and concentration, then subjected to DNA extraction for verification of X- and Y- chromosome bearing spermatozoa.

Sperm DNA Fragmentation

The unseparated and separated spermatozoa samples were assessed for the sperm DNA fragmentation. The analysis was performed using the bovine HALOMAX kit (HT-BT40, Spain) following the manufacturer's protocol. The spermatozoa were stained with 50 µg/ml of ethidium bromide and mounted in Vectashield antifading.

Primers and Probes Design

The genomic DNA was extracted from unseparated and separated caprine spermatozoa using a commercial DNA extraction kit (DNeasy Blood and Tissue Kit, QIAGEN Inc. Germany) according to the manufacturer's instruction manual. For the amplification, the primers and probes were developed to detect the X- and Y-chromosome bearing spermatozoa of *Capra hircus* based on ZFX and SRY genes, respectively using TaqMan qPCR. The forward and reverse primers, as well as probes, were drawn using the Beacon Designer software. The X-specific primers (forward: 5'-GAT AAG TTT ACA CAA CCA CCT GGA GAG-3' and reverse: 5'-CATGAG AGA AAT GCT TTC CGC-3') and the internal probe (5'-AGC AAG GCG GAG AAG-3') were designed according to GenBank accession number FJ349607.1 presenting a single fragment of 93 bp. Likewise, the Y-specific primers (forward: 5'-ACG ATG TTT ACA GTC CAG CGG-3' and reverse: 5'-TCC TGGCTG CTC TCC GTA AC-3') and the internal probe (5'-TTG TGC ACA GAC AAT CA-3') were designed based on GenBank accession number EU581862 which amplified a fragment of 137 bp. Both probes were labeled at the 5′ end with FAM fluorochrome. The probe system for each gene was double – dye oligonucleotide or TaqMan probe. The amount of PCR products or copy number of X- and Y- chromosome was detected when the dyes emitted fluorescent light that bound non-specifically to the sequence-specific probes.

Construction of Reference Plasmid DNA Clones

The ZFX and SRY genes were amplified by conventional PCR and cloned into pGEM-T vector (Promega, USA). The cloned products were subjected to plasmid extraction, qualitatively analyzed on 1% agarose gel before subjected to sequencing on the 3730xl DNA Analyser (Applied Biosystems, Courtabæuf, France). Analysis of the sequences showed that the identity of the genes was 99% homologous to ZFX and SRY genes in the GenBank database.

Quantitative Real Time PCR Using TaqMan Assay

The quantitative PCR was performed using StepOne^{TM}Plus Real Time PCR system (Applied Biosystem, USA). The PCR consisted of 10 µl reaction containing 0.5 µl of TaqMan Gene Expression Assay, 5 µl of TaqMan Fast Advance Master Mix 1X KAPA SYBR FAST qPCR Master Mix Universal (Kapa Biosystems, USA), 1 µl of DNA and 3.5 µl H_20. PCR reaction containing all the PCR mix but excluding the template was also included as a negative control. The PCR assay was run in triplicate for each DNA sample and primed in separate wells with single primer (either X- or Y-specific primer). The amplification was

performed in 0.1 ml MicroAmp® Fast 96-Well Reaction Plate (Applied Biosystems. USA) and the amplification consisted of enzyme denaturation for one cycle at 95 °C for 20 sec, followed by 40 cycles of amplification steps of denaturation at 95 °C for 3 sec and annealing at 60 °C for 20 sec. The amplification was ended with a disassociation step according to the instrument guidelines.

In order to gain precise calculation of the X- and Y-chromosome bearing spermatozoa in semen samples, a standard reference of plasmid clone for each gene was prepared. Five scalar 10-fold serial dilutions of each plasmid were performed over a range of copy number that included the amount of target DNA expected in the experimental samples. The initial concentration of each plasmid consisted of 10^{11} molecules. Standard curves were created by plotting the threshold cycle, C_T values vs log amount of DNA. The amplification efficiency was 99%, as determined by the R^2 value obtained from each standard curve. The standard curve generated from each clone of ZFX (R^2 > 0.993) and SRY (R^2 > 0.997) were used to estimate the copy number of X- and Y-chromosomes in the tested samples as described in the following equation:

Number of copies = (Amount x $6.022x10^{23}$) / (Length x $1x10^9$ x 650) = (ng x Number/mole) / (bp x ng/g x g/ mole of bp),

where, Amount is the amount of plasmid in nanograms (ng) and Length is the size of the plasmid in base pairs (bp). This calculation is based on the assumption that a single base pair has a molecular weight of 650 Da.

The percentage of X- and Y-chromosome bearing spermatozoa in semen samples was determined using the equation of Parati *et al.* (2006):

$$\% \text{ X chromosome} = \frac{n}{n+1} \times 100$$

$$\% \text{ Y chromosome} = 100\% - \% \text{ X chromosome}$$

where, n represents the ratio between the proportion of X and Y chromosomes in the semen sample.

Statistical Analysis

The data analysis was performed using SAS software, Version 9.3 of SAS Institute Inc. Cary, NC, USA. T-test was performed on the concentration and motility for separated spermatozoa in cathodic and anodic fractions. Differences between sex-separation treatments on percentages of fragmented and non-fragmented spermatozoa and percentages of X- and Y-chromosomes were determined using chi-square test. A probability of $P<0.05$ was considered significant for all statistical tests.

Results and Discussion

Fractions near the cathode had high motility rates (80.50 ± 2.29%) and number of spermatozoa (37.7 ± 16.87 $\times 10^6$ cell/mL) compared to anode (67.5 ± 6.11% and 7.73 ± 2.16 $\times$ 10^6 cell/mL, respectively) (Table 1). The concentration and motility (P_r = 0.0075, P_r <0.001) of spermatozoa was significantly lower ($P<0.05$) at the anode than the cathode. The semen sample was directly introduced to the electric field without washing and centrifuged to minimize prolonged exposure to the environment. The use of washed spermatozoa could improve the sex-separation efficiency but caused a reduction in spermatozoa motility (Manger *et al*., 1997). The motility rates of sexed spermatozoa could reach 90% and the number of spermatozoa was reasonably high. The

results were in general agreement with that reported by Manger *et al*. (1997), where it was shown that cell motility varied from 85% in a fraction near the cathode to 4 to 10% near the anode. The motile spermatozoa were observed to exhibit galvanotactic migration toward the cathode (Nevo *et al*., 2004; Blottner *et al*., 1994). However, Masuda *et al*. (1989) found that the most motile bovine spermatozoa were near the anode rather than the cathode.

Table 1: Concentration and motility of separated spermatozoa in cathodic and anodic fractions

Sex-separation treatment	Concentration (cell/mL)	Motility (%)
Separated spermatozoa (cathode)	$37.7 \pm 16.87 \times 10^6$	80.50 ± 2.29
Separated spermatozoa (anode)	$7.73 \pm 2.16 \times 10^6$	67.5 ± 6.11

DNA fragmentation can be thought of as a marker for other types of damage to the sperm. By using the Halomax kit the spermatozoa with non-fragmented and fragmented DNA produced the small and large halo zone, respectively around the head (Figure 1). The fragmented spermatozoa in cathodic and anodic fractions were 51.2 % and 47.83 %, respectively compared to unseparated spermatozoa 46.57 % (Table 2). From the analysis, it shows that this sex-separation procedure did not cause damage to spermatozoa (P>0.05).

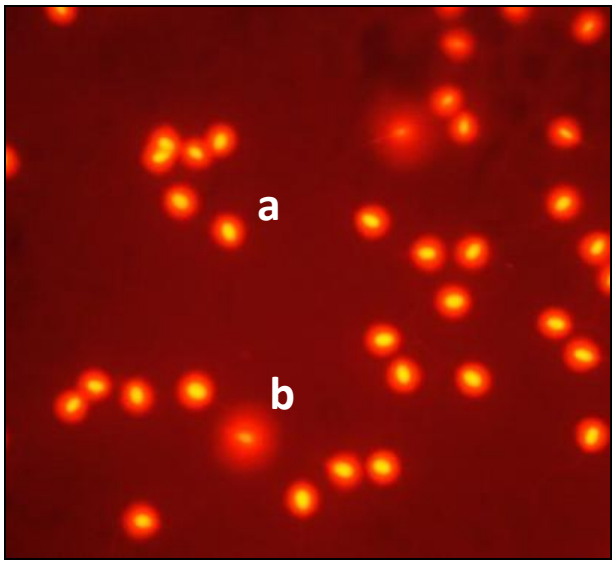

Figure 1: Analysis of sperm fragmented DNA visualized under fluorescence microscope (X63).
a: non-fragmented spermatozoa (small halo),
b: fragmented spermatozoa (large halo)

Table 2: Percentage of non-fragmented and fragmented DNA in samples of unseparated and separated spermatozoa using qPCR

Treatment	Non-fragmented spermatozoa	Fragmented spermatozoa
Unseparated spermatozoa	53.45 ± 3.27[a]	46.57 ± 3.37[a]
Separated spermatozoa (cathode)	49.62 ± 4.08[a]	51.2 ± 4.94[a]
Separated spermatozoa (anode)	57.48 ± 5.71[a]	47.83 ± 6.12[a]

[a]Percentages within rows with similar superscripts are not significantly different (P>0.05)

A quantitative real-time PCR (qPCR) analysis was performed to validate the spermatozoa populations in both anodic and cathodic fractions, as well as to determine the accuracy of the free-flow electrophoresis spermatozoa sex-separation technique. The ZFX and SRY genes were successfully amplified in semen DNA samples presenting a single fragment at size 93 bp and 137 bp, respectively. The plasmids containing ZFX and SRY genes were used as a standard reference to obtain two regression equations to estimate the copy number of ZFX and SRY genes in the tested samples (Figure 2).

The principles and quantification of real time PCR were based on a threshold cycle (C_T value) of respective samples. The threshold cycle is the cycle number (C) at which the fluorescence crossed a threshold (T). The threshold is a fluorescence value that is slightly above the background fluorescence measured before exponential growth starts (Bubner and Baldwin, 2004). The low value of C_T indicates a high target amount of gene copy number and if high values the opposite (Figure 2). The interpolation of the C_T_X and C_T_Y values that resulted from the analysis of semen samples, on the fluorescent plots (Ampl-X and Ampl-Y) shown by Figure 3 was obtained from the amplification of X- and Y-chromosome targets of unseparated and separated semen samples. The fluorescent signal reached a threshold level that correlated with the amount of original target sequence, thereby enabling quantification. The intercepts between the threshold and the plots allowed computing the C_T_X and C_T_Y.

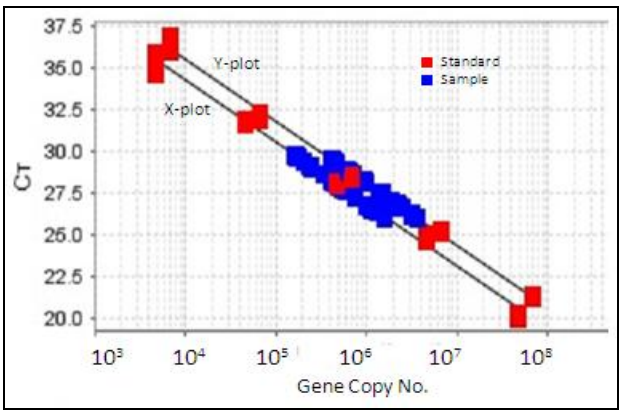

Figure 2. Linear regression plots (X-plot and Y-plot) generated by plotting C_T values of ZFX and SRY gene fragments inserted into the plasmids against each dilution point (red box).

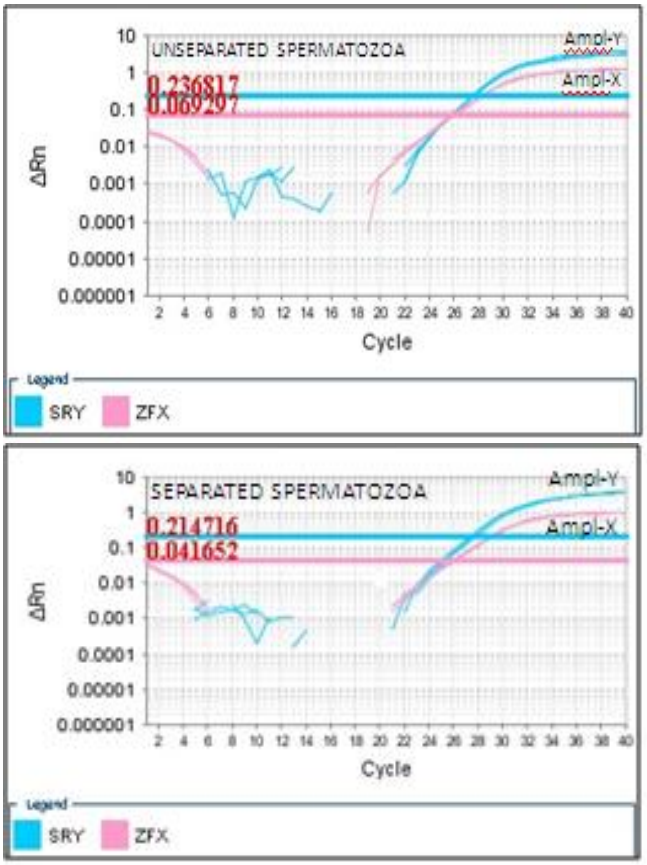

Figure 3: The fluorescent plots (Ampl-Y and Ampl-X) obtained from the X- and Y-chromosome targets from representative
i) unseparated spermatozoa ii) separated spermatozoa

Determination of X- and Y-chromosomes in semen samples was calculated following the equation described by Parati *et al*. (2006). Verification of X- and Y- chromosome bearing spermatozoa in unseparated spermatozoa by qPCR based on ZFX and SRY genes was 51 and 49%, respectively (Table 3). The unseparated spermatozoa always ran together with separated spermatozoa during PCR assay. The results were in general agreement with the previous report where it was shown that the X : Y sperm ratio was 1:1 (Garner *et al*., 1983; Vidal *et al*., 1993; Rose and Wong, 1998; Tan, 2009 and Maleki *et al*., 2013). However, Delgado *et al*. (2010) reported that the ratio of X- and Y-chromosome skewed from the expected 1:1 ratio following verification protocols of Parati *et al*. (2006). Disequilibrium in the proportion of X- and Y- chromosome bearing spermatozoa could be due to environment and physiological factors (Hardy, 1997) or the time of insemination during estrus (Gutierrez *et al*., 1999). However, the skewness in sex ratio could be due to inaccuracy of verification method and variation in expected ratio. In the present study, verification of separated spermatozoa by free flow electrophoresis in cathodic fraction showed that the X- and Y-chromosomes was 36:64, and in anodic fraction 44:56, respectively, and were not significantly different (P>0.05) compared to unsexed-separated spermatozoa, 51:49 (Table 3). Sureka *et al*. (2013) made an effort to select Y-chromosome bearing spermatozoa in Sannen and Jamnapari goats through low cost method of sucrose gradient and was able to separate Y-chromosome for about 62.6% in Jamnapari and 59.0% in Sannen at 1% sucrose gradient layer. However, the efficiency of this simple method was low compared to flow cytometer which could separate semen samples into two populations with purity 85 - 95 % (Maxwell *et al*., 2004). Prasad *et al*. (2010) reported that the X- chromosome having higher negative charge than Y-chromosome migrated towards anode. Manger *et al*. (1997) emphasized that the separation of bovine semen by free flow electrophoresis indicated enrichment of Y-chromosome bearing spermatozoa. Masuda *et al*. (1989) found that bovine Y-chromosome was enriched in anodic fractions and X-chromosome in cathodic fractions; however Blottner *et al*. (1994) showed an opposite enrichment. From these studies, the migration routes of spermatozoa were shown to be different between inter- and intra-species (Engelmann *et al*., 1988). Thus, at this juncture, the migration characteristics of caprine spermatozoa to the cathode and anode are not clear.

Table 3: Percentage of X- and Y-chromosome in samples of unseparated and separated spermatozoa using qPCR

Treatment	Chromosome type	
	X-chromosome	Y-chromosome
Unseparated spermatozoa	50.74 ± 0.78[a]	49.26 ± 0.78[a]
Separated spermatozoa (cathode)	36.02 ± 3.12[a]	63.98 ± 3.12[a]
Separated spermatozoa (anode)	44.27 ± 6.01[a]	55.7 ± 6.01[a]

[a]Percentages within rows with similar superscripts are not significantly different (P>0.05)

Previously, the Fluorescence *in situ* hybridization (FISH) has been used effectively for validation of sperm sexing (Wang *et al*, 1994; Classens *et al*., 1995; Flaherty *et al*., 1997; Habermann *et al*., 2005). However, this technique has its own limitations. The FISH takes at least three days to complete coupled with problems of photo bleaching, tedious, and time consuming. Furthermore, FISH can verify only a smaller quantity of X- and Y-chromosomes bearing spermatozoa in a semen sample. Therefore, this technique is not appropriate to be used as a routine validation for sperm sexing. The development of TaqMan PCR assay was shown to be an accurate tool for estimating sex chromosome composition in caprine semen sample. This technique could be used as a routine verification of sexed chromosome as well as to determine the efficiency of sex-separation techniques.

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