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Goat Polyclonal Antibody Against the Sex Determining Region Y to Separate X- and Y-Chromosome Bearing Spermatozoa

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Abstract

Background: Sex selection of sperm by separating X- and Y-chromosome bearing spermatozoa is critical for efficiently obtaining the desired sex of animal offspring in the livestock industry. The purpose of this study was to produce a goat polyclonal antibody (pAb) against the bovine Sex Determining Region Y chromosome (bSRY) to separate female- and male-bearing spermatozoa.

Methods: To produce a goat polyclonal antibody against bSRY, a female goat was subcutaneously immunized with 27 kDa of recombinant bSRY (rbSRY) protein as the antigen. The anti-bSRY pAb was purified by ion-exchange chromatography. The purity of the pAb was determined using the SDS-PAGE method. The biological activity of the anti-bSRY pAb was examined using PCR to assess the binding affinity of pAb for the bSRY antigen and commercially sexed bull sperm.

Results: The total amount of purified anti-bSRY pAb was approximately 650 mg/goat serum (13 mg/mL). Interestingly, our data showed that the binding affinity of our pAb to the Y bearing was high, while the binding affinity of that to the X-chromosome bearing sperm was similar to the negative control.

Conclusions: In conclusion, our findings show that the goat anti-SRY pAb specifically binds to Y-chromosome bearing sperm that suggesting its potential use for sex selection.

Keywords: Polyclonal antibody, Sex determining region Y chromosome, Sperm sexing.

Introduction

The pre-selection of the sex of sperm in the dairy and meat cattle industry permits higher productivity, faster genetic selection, and reduces the frequency of dystocia in heifers. Controlling the sex ratio also improves animal welfare, reduces the overall environmental impact of farming, and allows for the specific selection of female offspring for milk production and male offspring for meat production (1, 2). Current techniques for the separation of X- from Y-chromosome bearing spermatozoa are based on sperm swimming patterns, differences in mass and motility, DNA content, size, weight, electrical surface charge, centrifugal countercurrent distribution, volumetric differences, and immunologically approaches, the later method relatively more specific than the other methods (3-5). Methods for the separation of X- and Y-chromosome bearing spermatozoa include differential separation through Percoll (6) and albumin gradients (6, 7), the modified upswing-up method(8), flow cytometry (9),and techniques taking advantage of the sex-specific antigen (SSA) or sex chromosome-specific protein (SCSP) (10, 11), offspring sex-specific antigens (OSSAs) (12), and the H-Y antigen (13). Previous studies using the fluorescence *in situ* hybridization (FISH) approach indicated that the albumin gradient (14, 15), sephadex column (16), and swim-up techniques (17, 18) did not sufficiently separate X- and Y-spermin humans, while the discontinuous percoll gradient showed high efficiency separation (19, 20).

Kobayashi et al. (2004)(21)and Lima et al. (2006)(22) used centrifugation on a discontinuous Percoll density gradient as a method of separating

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X- and Y-sperm. Their results showed the separation ratio of X- to Y-chromosome bearing sperm in bovine semen samples to be 55.7% and 74.3%, respectively. Asma-ul-Husna et al. (2017) used a modified swim-up method for sperm sex selection and showed that this approach effectively separated X- from Y-chromosome bearing spermatozoa. To validate the sexed sperm, real time PCR was used to determine the expression of PLP gene for the X-chromosome and SRY gene for the Y-chromosome (23).

The immunological methods for the sex selection of sperm is based on the differential expression of proteins found of the surface of X- and Y-chromosomes bearing sperm. Immunological approaches have been applied to identify sperm antigens that bind to the zona pellucida (24), that can detected capacitation of related antigens (25). Sang et al. (2011) (26) attempted to isolate X- and Y-chromosome bearing spermatozoa via immunological methods by exploiting sex-specific proteins (SSPs). However, the sex-specific antigen was demonstrated only by flow cytometry.

In most mammalian species, sex is specified by the presence or absence of the Y chromosome. The X and Y chromosomes encode for specific proteins that can be used for sperm sexing. The sex determining region Y (SRY) is an intron-less gene located on the Y chromosome (27). The theory behind this means of sperm sexing is that antibodies developed against SRY can separate Y- from X-chromosome bearing spermatozoa. However, no reports are currently available indicating whether anti-SRY antibodies can be successfully used for sperm sexing (28). In bovine species, SRY is transcribed to yield a protein with 229 amino acids (29).

In the present study, we aimed to produce a polyclonal antibody (pAb) against recombinant bovine SRY (rbSRY) for the separation of X- and Y-chromosome bearing spermatozoa. The specific binding of anti-rbSRY pAb to commercially sexed Y-chromosome bearing bull sperm was evaluated.

Materials and methods

Experimental animal

A six-month old female goat (Razi Biotech. CO, Kermanshah, Iran) was maintained in specific pathogen free (SPF) conditions. All animal experiments were performed in accordance with the Animal Care and Use Protocol of Kermanshah University of Medical Sciences.

Goat Immunization

To produce the anti-rbSRY pAb, a female goat was subcutaneously immunized with purified rbSRY produced as previously described (30, 31). The goat was immunized four times by subcutaneously injection of 650-750 µg of rbSRY in Complete Freund's adjuvant (CFA, Sigma-Aldrich) for the first injection and Incomplete Freund's adjuvant (IFA, Sigma Aldrich) for the subsequent three booster injections for two weeks' interval. One week following the last immunization, a blood sample was obtained from the jugular vein in heparinized tubes. Goat sera titration was examined via indirect ELISA for the detection of anti-rbSRY antibodies. For this assay, rbSRY was coated onto ELISA plate wells for 1 h at room temperature. Wells were washed (BioHIT, EL x50) three times with PBS containing 0.05% tween 20 followed by blocking with 200 µL of 1% BSA and 0.1% tween 20 in PBS for 45 min at room temperature. The goat serum was diluted to various concentrations (1:4000, 1:8000, 1:16000, 1:32000, 1:64000, 1:128000 and, 1:256000). The serum was then added to the wells and incubated for 45 min at room temperature in duplicate. The wells were then washed three times with PBS containing 0.05% tween 20 followed by incubation with HRP-conjugated rabbit anti-goat IgG (Razi Biotech, Kermanshah, Iran) at a dilution of 1/50000 in PBS with 2% BSA and 0.1% tween 20 for 1 h at room temperature. The wells were then washed six times with PBS containing 0.05% tween 20. A tetramethylbenzidine substrate solution (TMB, 100 µL) (Sigma -Aldrich) was added for the visualization of the antigen-antibody reaction. The plates were incubated for 10 min in the dark. The reaction was stopped with 50 µL of 2 M sulfuric acid and the optical density (OD) was read at a wavelength of 450 nm using a multi-well plate reader (AWARNESS, Stat fax-2100).

Production and purification of goat anti-rbSRY pAbs

For the production of the goat anti-rbSRY pAbs from serum, the IgG fraction of the isolated goat

serum was precipitated with semi-saturated ammonium sulphate (50%). The precipitated fraction was then dialyzed against PBS, pH 7.5, and applied ion exchange column (GE Healthcare Bio-Sciences AB). The column was washed thoroughly with PBS containing 0.35 M NaCl (pH 7.5). The captured antibodies were eluted from the column with a solution of 0.1 M glycine-HCl and 0.5 M NaCl (pH 2.7) and neutralized with 1 M Tris-HCl, pH 8, followed by dialysis against PBS. The concentration of the purified pAb was determined by NanoDrop 2000c (Thermo Fisher Scientific, Waltham, MA). The purity of the anti-rbSRYpAb was assessed by SDS-PAGE, followed by Coomassie Blue R-250 staining.

Assessing the binding affinity of goat anti-rbSRY pAb with rbSRY by ELISA

After purifying the goat anti-rbSRY pAb from the serum using ion exchange chromatography, the pAb titers were determined by an indirect ELISA method. Each well of the 96-well microtiter plate was coated with 100 µl of 3 µg/mL rbSRY protein in carbonate buffer and incubated at room temperature for 2 hours. After two washes with PBS and blocking with 200 µL of 1% BSA and 0.1% tween 20 in PBS for 45 min at room temperature, 100 µl of the anti-rbSRY pAb was tested at 1/2000, 1/4000, 1/8000, and 1/16000 dilution in 1% BSA. The plate was covered with an adhesive plastic and incubated for 2 h at room temperature, then washed six times with PBS. Next, 100 µl per well of 1:5000 diluted horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG (Razi Biotech). CO, Kermanshah, Iran) was added and incubated for another 45 min at room temperature. Following this, 100 µL of TMB substrate solution was added in the dark. After adding 50 µL of stop solution to the wells, the absorbance was read at a wavelength of 450 nm with a micro plate reader (AWARNESS, Stat fax-2100).

Assessing the affinity of the goat anti-rbSRY pAb against commercially sexed bull sperm

The binding affinity of the goat anti-rbSRY pAb against5 different commercially sexed bull sperm samples were evaluated via ELISA. In this experiment, each well of the 96-well micro-titer plate was coated with 100 µl of 300 µg/mL of pAb in carbonate buffer and incubated at room temperature for 3 hours. After two washes with PBS and blocking with 200 µL of 1% BSA and 0.1% tween 20 in PBS for 1 hour at room temperature, 100 µl of the commercially sexed bull sperm in 1% BSA was tested at either 0, 50, 100, 200, or 400 thousand cells per well. The plate was covered with an adhesive plastic and incubated for 1 h at room temperature, then washed six times with PBS. Next, 100 µl per well of 1:5000 diluted horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG (Razi Biotech. CO, Kermanshah, Iran) was added and incubated for 30 min at room temperature. Following this, 100 µL of TMB substrate solution was added in the dark. After adding 50 µL of stop solution into the wells, the absorbance was read at a wavelength of 450 nm with a microplate reader.

PCR amplification of the SRY gene in Y-chromosome bearing spermatozoa

We hypothesized that our goat anti-rbSRY pAb is capable of binding Y-chromosome bearing spermatozoa with high affinity and is unable to bind X-chromosome bearing spermatozoa due to its specificity for SRY. To test this hypothesis and examine the binding affinity of our goat anti-rbSRY pAb, PCR was used. The 690 bp ORF sequence of bSRY was selected from the NCBI database (accession number: NM-001014385.1). For DNA extraction from the commercially available bull sperm samples, the modified salting out method was used and a SRY gene synthesis was done using PCR (Eppendorf Germany) and specific primers. The forward and reverse primers were (5' to 3) ATGTTCAGAGTATTGAACGA and (5' to 3) TCAATATTGAAAATAAGCAC. The materials in the PCR reaction tube included 2.5 µL 10 X *Pfu* amplification buffer, 1.5 µL 10 mM dNTPs mixture, 1 µL 50 mM MgSO4, 2 µL primer mix (10 pm), 0.5 µL sperms template DNA (350 ng/µL), 0.5 µL *Pfu* DNA polymerase (M7741, Promega, Madison, USA) and, 17 µL ddH2O. Thermal cycling conditions were 94 °C for 5 min followed by 32 cycles of 94 °C for 1 min, 58 °C for 45 sec, 72 °C for 1 min and then a 10 min final extension at 72 °C. Afterwards, PCR products were examined by gel electrophoresis

in 1% (w/v) agarose gel using a 1 kb DNA marker. We conducted PCR on the samples eluted from the column following the addition of X-chromosome bearing spermatozoa into the column.

Results

Production and purification of goat anti-rbSRY pAbs

Serum antibody titers were analyzed by indirect ELISA (Table 1). The serum (50 mL) was isolated from the blood sample and treated with ammonium sulphate (50% v/v), then purified by ion exchange chromatography to obtain the desired IgG fraction. The total amount of purified pAb was approximately 650 mg/goat serum (13 mg/mL). The purity of the pAb was confirmed by SDS-PAGE and Comassie Blue staining. The purified pAb under reducing conditions, two bands of the expected sizes for the immunoglobulin light (25 kDa) and heavy chains (50 kDa) were detected (Fig. 1).

Table 1. Serum of the goat immunized with rbSRY was assayed by ELISA at OD 450_{nm} (Avg ± SD).

Goat serum	Control	1:8000	1:16000	1:32000	1:64000	1:128000	1:256000
1	0.14±0.03	3.21±0.11	2.69±0.08	1.85±0.19	0.98±0.14	0.7±0.09	0.61±0.08
2	0.17±0.04	3.53±0.1	2.7±0.13	1.81±0.14	1.11±0.12	0.69±0.08	0.58±0.1
3	0.16±0.02	3.61±0.09	2.66±0.11	1.74±0.17	0.97±0.14	0.62±0.11	0.53±0.11

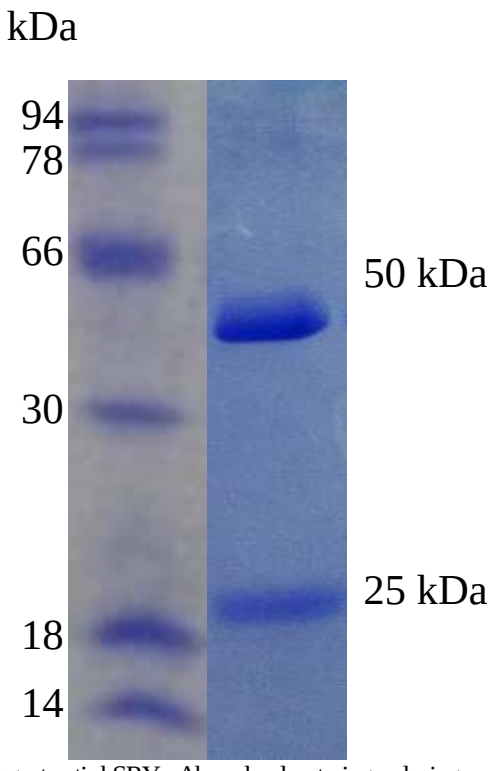

Fig. 1. The SDS-PAGE analysis of purified goat anti-rbSRY pAb under denaturing-reducing condition showed two bands, one at 50 kDa (heavy chain) and another at 25 kDa (light chain).

Binding affinity of SRY pAb to rbSRY antigen

The binding affinity of the goat anti-bSRY pAb to rbSRY was analyzed using an indirect ELISA. With increasing pAb concentrations, the binding affinity of the rbSRY antigen to the pAb was observed to increase (Table. 2).Given the purification strategies carried out for rbSRY in the last our previous studies (30, 31), this protein had the highest purity and its binding to mouse anti-rbSRY pAb was specific.

Table 2. The binding affinity of goat anti-bSRY pAb to the rbSRY antigen at 450 nm (Avg. ±SD).

Average pAb-rbSRY binding (±SD)	Control	1:2000	1:4000	1:8000	1:16000
	0.19±0.09	3.62±0.18	2.14±0.11	1.55±0.09	0.89±0.11

Binding affinity of the goat anti-rbSRY pAb to commercially sexed spermatozoa

Additionally, we evaluated the potential of the purified pAb to specifically bind to Y-chromosome bearing spermatozoa. Our findings indicate that only Y-chromosome bearing spermatozoa (semen 3, 4, and 5) were able bind to the goat pAb, while the X-chromosome bearing spermatozoa (semen 1 and 2) were unable to bind the pAb. This phenomenon was shown by the high 450 nm absorbance of Y-chromosome sperm to the pAb (Table 3). Our results showed that the OD 450 observance of X-chromosome bearing spermatozoa to be near the level of the negative control sample.

Table 3. The binding affinity of the goat anti-rbSRY pAb against commercially sexed bull sperm samples (Ave.±SD).

Control	Semen 1	Semen 2	Semen 3	Semen 4	Semen 5
0.25±0.05	3.3±0.22	3.4±0.44	0.21±0.02	0.23±0.02	2.87±0.27

PCR amplification of the bSRY gene in Y-chromosome bearing spermatozoa

After determining the binding affinity of the anti-rbSRY pAb to the Y-chromosome bearing spermatozoa (semen 1, 3, and 5), PCR was performed to detect the presence of the SRY gene in these semen samples (Fig. 2). Since the SRY gene is located on the Y chromosome, detection of this gene in these sperm samples confirm that the high binding affinity of the pAb is in fact against the Y-chromosome bearing spermatozoa. The PCR results indicated the presence of a 690 bp band corresponding to bSRY in the Y-chromosome bearing spermatozoa, while this band was absent after performing PCR on the samples containing the X-chromosome bearing spermatozoa (semen 1 and 2).

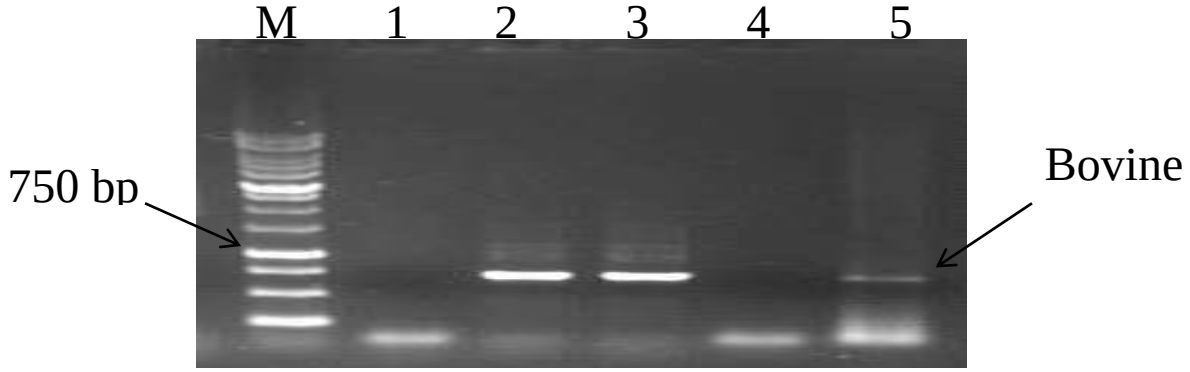

Fig. 2. Gel electrophoresis of SRY PCR products from 5 different samples of bull sperm. Lanes 1 and 4: PCR products of X-chromosome bearing spermatozoa, M: 1000 bps DNA standard marker, lanes 2, 3, and 5: PCR products of Y-chromosome bearing spermatozoa that contain the SRY gene.

Discussion

In the present study, we produced a goat anti-rbSRY pAb for the separation of X- and Y-chromosome bearing spermatozoa. The role of SRY in the sex selection of sperm has not yet been investigated. Selection of the desired sex of farm animals, particularly in meat and dairy cattle, has attracted considerable attention. Due to the biological and economic disadvantages of flow cytometry including; using the florescent dye, happen the mutation in some of sexed sperms, fragile sperm motility, change the morphology of sexed sperms and cost benefit of this technology, the other methods including albumin gradients, modified swim-up methods, and methods

exploiting SSA or SCSP have been developed for sperm sexing. These approaches are based on the density, motility, surface charge, and sex-specific proteins of X- and Y-chromosome bearing spermatozoa. Azizeddin et al. (2014) reported a swim-up method E capable of separating X- and Y-chromosome bearing spermatozoa (32). However, the ability of this swim-up method to successfully determine the sex of spermatozoa in all species was relatively low, necessitating the development of more accurate and efficient techniques. The immunological method of spermatozoa sex selection is based on the presence of specific proteins on X- and Y-chromosome bearing spermatozoa that distinguish them from one another. Since the SRY gene is specifically located on the Y chromosome, our hypothesis predicts that by producing a pAb against the SRY protein we can separate Y-chromosome bearing spermatozoa from X-chromosome bearing spermatozoa. Although flow cytometry is the most reliable method for sperm sexing (33), this method also contains several drawbacks including: high cost, the use of the Hoechst 33342 dye which can damage DNA of the sorted spermatozoa, and the flow cytometer requires an expert operator. Therefore, better and more accessible methods of sperm sorting should be considered. In humans, researchers have shown that the eight-step discontinuous gradient is not a reliable method for the separation of X- and Y-chromosome bearing spermatozoa due to its low efficiency(34). Given the presence of specific proteins unique to X- and Y-chromosome bearing spermatozoa, we can develop immunological approaches including those that exploit pAb technology for sperm sorting. In our study, we used commercially sexed X- and Y-chromosome bearing bull sperm to show the specific binding of our goat anti-rbSRY pAb. According to our findings, the goat anti-rbSRY pAb is capable of specifically binding to Y-chromosome bearing spermatozoa while it does not bind X-chromosome bearing spermatozoa. This specificity indicates the Y-chromosome specific expression of SRY. Additionally, our study surprisingly demonstrates surface expression of SRY (or at least some of its fragments or epitopes) on Y-chromosome bearing spermatozoa which suggest a potential role for surface SRY in fertilization. However, this should be further explored and requires additional investigation to be confirmed. Though indirectly (through SRY2mAb-coupled affinity column capture of Y sperms) inferred, our study provides the first known evidence on the surface expression of SRY (or at least some of its fragments) on Y-chromosome bearing spermatozoa. The gradient methods for the separation of X- and Y-chromosome bearing spermatozoa are safe, available, easy, and cost-effective, but the efficiency of these approaches is relatively low compared to immunological assays such as those using monoclonal antibodies against X- or Y-chromosome specific proteins. The methods such as the swim-up method(35), Percoll(7) and albumin gradients(36),are used routinely for farm animals and human to obtain a population of highly motile spermatozoa for many assisted reproductive techniques(ART) such as artificial insemination (AI),*in vitro* fertilization (IVF), and embryo transfer. Flow cytometry, quinacrine dye, polymerase chain reaction (PCR) and fluorescence in situ hybridization (FISH) techniques have been used to confirm accurate sperm sexing (37-41). Among these, the PCR and FISH techniques have been shown to provide the most reliable results (42, 43). PCR is a labor intensive technique used for screening a large number of sorted X- and Y-chromosome bearing spermatozoa (44). In the present study, we used the PCR method to confirm the efficiency of our goat anti-SRY polyclonal antibody in separating Y- from X-chromosome bearing spermatozoa.

We produced a goat anti-rbSRY pAb and analyzed its binding affinity to commercially sexed X- and Y-chromosome bearing bull sperm. The results obtained from our study revealed a high reactivity of our pAb against Y-chromosome bearing sperm compared to X-chromosome bearing sperm. Although commercially sexed Y-chromosome bearing sperm reacted with the pAb, future experiments should explore how our pAb binds to whole bull semen samples. Additionally, the viability as well as functional parameters of the eluted sperm (such as motility) were not assessed and will be examined in future research. Our results show that our goat anti-rbSRY pAb can accurately distinguish between Y- and X-chromosome bearing spermatozoa, shedding light on its potential for developing immunologic-based approaches such as

immune affinity chromatography for sperm sexing and sorting.

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