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Sexing single bovine blastomeres using *TSPY* gene amplification

M.C.A. Carneiro[1,2], P.L. Takeuchi[1], A. Araújo[1], R.B. Lôbo[1], F.P. Elias[1], R.A. Vila[1], C.L. Miranda-Furtado[1] and E.S. Ramos[1,3]

[1]Departamento de Genética, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil
[2]Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil
[3]Departamento de Ginecologia e Obstetrícia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil

Corresponding author: E.S. Ramos
E-mail: esramos@genbov.fmrp.usp.br

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ABSTRACT. The testis-specific protein Y-encoded gene (*TSPY*) is a Y-specific gene present in variable copy number in many mammalian species, including cattle. We tested the applicability of the *TSPY* gene as a Y-specific marker to predict preimplantation embryo sex in Nelore (*Bos indicus*) cattle. Two blastomeres were removed from each embryo. A total of 36 single blastomeres and the remaining cells of their 18 matched *in vitro* conceived embryos were screened for *TSPY* amplification by nested-PCR. The results obtained from a single blastomere and the remaining cells of the same embryo were concordant in all cases. All blastomeres (16/16) from eight embryos produced with sexed sperm (specific for production of male embryos) were *TSPY*-positive. We conclude that *TSPY* is a good male-specific marker, the usefulness of which is probably enhanced by the high copy number. Other methods that are less time-consuming, such as real-time

PCR, could be improved with the use of the *TSPY* gene sequences to generate primers and/or probes. This is the first report to demonstrate the applicability of the *TSPY* gene for sexing single cells in cattle.

Key words: Sexing; *TSPY* gene; Bovine; Embryo; Single cell; Cattle

INTRODUCTION

The identification of bovine embryo gender can be very useful for research involving differential expression of genes between males and females (Chrenek et al., 2001; Bermejo-Alvarez et al., 2008). Similarly, it can be a valuable tool for assisted reproduction in breeding programs, and may also be commercially explored in the cattle industry.

Many techniques and Y-specific markers have been used for sexing cattle (Lee et al., 2004; Bermejo-Alvarez et al., 2008; Zoheir and Allam, 2010). The application of polymerase chain reaction (PCR) for the analysis of a single cell presents a challenge since limited DNA is available in one single diploid cell, and this can lead to a reduction in amplification efficiency. Lemos et al. (2005) described a study on the *TSPY* (testis-specific protein Y-encoded) gene as a male-specific marker for cattle sexing in very low DNA concentrations. The *TSPY* gene is advantageous compared to other markers because it is a Y-specific gene of multiple copies both in human and bovine genomes. After embryo biopsy, the DNA from only one cell is amplified, so, using genes with multiple copies, we could increase the efficiency of the amplification (Bartmann et al., 2004; Lemos et al., 2005).

In the present study, we tested a nested PCR-based protocol, modified from Lemos et al. (2005), in order to sex single cells from bovine preimplantation embryos using a sequence of the *TSPY* gene (also known as *DYS14* in humans).

MATERIAL AND METHODS

Bovine embryos

Immature bovine cumulus-oocyte complexes (COCs) were aspirated from 2- to 7-mm follicles in the ovaries of slaughtered cows (Nelore cattle - *Bos indicus*). The selected oocytes were washed twice in H199+ medium and twice in B199+ maturation medium. COCs were cultured in 5% CO_2 at 38.7°C for 22-24 h for maturation, in B199+ covered with mineral oil. The semen straws from Nelore bulls (*Bos indicus*) were thawed, and the semen was separated on a Percoll (GE) gradient. After the maturation period, the COCs were washed twice in the *in vitro* fertilization (IVF) medium, and co-incubated with the motile spermatozoa in microdrops of 100 µL (90 µL IVF medium + 10 µL spermatozoa pellet diluted in IVF medium to a concentration of 2.0 x 10^6 spermatozoa/mL) covered with mineral oil. The oocytes and spermatozoa were co-incubated for 12-15 h in 5% of CO_2 at 38.7°C.

After the fertilization period, the zygotes were washed in IVF medium and three times in CR2+ medium. They were co-cultivated in CR2+ medium for 72 h. After this period, the viable embryos (8-16 cells) were washed and transferred to microdrops with manipulation medium, and, using a micropipette 10 µm in diameter coupled to a micromanipulator (Narishigi Co. Ltda., Tokyo, Japan), the embryos were fixed. With the biopsy pipette (35-µm diameter),

the zona pellucida was broken, and a single blastomere was removed. From each embryo, at least two blastomeres were individually removed. Each single blastomere and the rest of the embryo were individually stored in tubes at -80°C.

Molecular analysis

In the present study, DNA extracted from 18 biopsied embryos (10 obtained by IVF using non-sexed semen, and 8, by IVF using sexed sorted semen, specific for generating male embryos) were analyzed. We carried out a thermal-shock DNA extraction and 1 µL proteinase K (25 mg/mL) and 49 µL 0.005% SDS were added. The tubes were incubated in a water-bath at 55°C for 1 h, and incubated in a dry-bath for 30 min at 99°C. The tubes were immediately transferred to ice, and the PCR solution was added.

The sequences of the primers, based on sequences reported in other studies (GenBank No. X74028.1, *B. taurus TSPY* gene) (Jakubiczka et al., 1993), were as follows: forward primer (Ext *TSPY* S) 5'-CCCGCACCTTCCAAGTTGTG-3', reverse primer (Ext *TSPY* AS) 5'-TGCTCCTC CACCGTCTTCT-3'. The second set of primers was: 5'-TTGTCACCAGCAGTTGTCACG-3' (Int *TSPY* S) and 5'-AACCTCCACCTCCTCCACGATG-3' (Int *TSPY* AS).

The first PCR contained 12.05 µL MilliQ water, buffer solution with 1.5 mM $MgCl_2$ Crimson *Taq* reaction buffer (10 mM Tris-HCl, 100 mM KCl, 0.1 mM EDTA, 1mM dithiothreitol, 0.5% Tween 20, 0.5% NP-40 and 50% glycerol) (New England BioLabs Inc.), 0.1 mM of each dNTP (Invitrogen), 10 pmol of each external primer (forward and reverse), 0.625 U Crimson *Taq* DNA polymerase and 8 µL lysis solution containing all the extracted DNA. The second PCR consisted of 22 µL MilliQ water, buffer solution with 1.5 mM $MgCl_2$ Crimson *Taq* reaction buffer (New England BioLabs Inc.), 0.1 mM of each dNTP, 10 pmol of each internal primer (forward and reverse), 0.625 U Crimson *Taq* DNA polymerase and 3 µL first PCR product.

The DNA sequence was amplified in a T Gradient (Biometra) thermocycler by an initial denaturation step at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 45 s, annealing at 60° and 64°C (first and second PCR, respectively) for 45 s and elongation at 68°C for 45 s. An additional extension time of 10 min at 10°C was added at the end of the final cycle. The amplification products (328 bp for the first set of primers, and 153 bp for the second) were analyzed by electrophoresis in 8% polyacrylamide gels stained with 2% silver nitrate. As a DNA positive control, we used a bovine *β-actin* gene sequence previously described (Rios et al., 2007).

We compared the results of each single blastomere (in a total of two blastomeres from each embryo) with the data obtained from the other blastomere and the remaining cells of the same embryo. Each reaction contained DNA extracted from testis (male control) and cow blood samples (female control) as controls.

RESULTS

The results are illustrated in Figure 1 and summarized in Table 1. For all embryos, the results of the three samples (two blastomeres and the remaining cells of the embryo) matched. All sexed semen-derived embryos showed results compatible with male embryos, as expected.

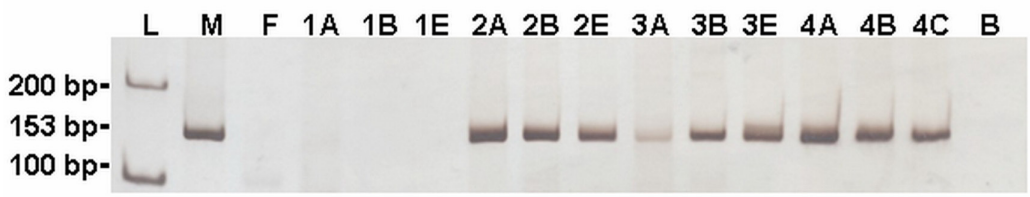

Figure 1. Results of polyacrylamide gel electrophoresis with silver nitrate staining. When the band (153 bp) was present, the result was considered *TSPY*-positive. (L) molecular marker (100 bp); (M) testicular tissue or male control; (F) cow's blood or female control; (1-4) embryos numbered according to Table 1; (A and B) single blastomeres; (E) remaining cells of the matched embryo; (B) negative control without DNA.

Table 1. Gender determination of single blastomeres and matched embryos by nested PCR, using bovine *TSPY* gene specific primers.

Embryos	Blastomere 1	Blastomere 2	Remaining cells	Gender
	TSPY	*TSPY*	*TSPY*	
Non-sexed semen				
1	-	-	-	Female
2	+	+	+	Male
3	+	+	+	Male
4	+	+	+	Male
5	+	+	+	Male
6	-	-	-	Female
7	+	+	+	Male
8	+	+	+	Male
9	-	-	-	Female
10	+	+	+	Male
Sexed semen				
11	+	+	+	Male
12	+	+	+	Male
13	+	+	+	Male
14	+	+	+	Male
15	+	+	+	Male
16	+	+	+	Male
17	+	+	+	Male
18	+	+	+	Male

DISCUSSION

The genomic *TSPY* organization of the bovine and human genes is highly conserved (Vogel et al., 1997). In *Bos taurus*, most breeds have a similar average *TSPY* copy number (94 copies) (Hamilton et al., 2009). In a previous study, we reported that PCR using primers derived from a *TSPY* sequence allows the detection of the Y chromosome in male animals (Nelore cattle) even at low DNA concentrations. These preliminary results showed the potential of this gene as a male-specific marker (Lemos et al., 2005).

In the present study, we were able to identify embryo sex in a quick and specific way, using a single biopsied blastomere and a nested PCR with the *TSPY* gene.

Despite the fact that the accuracy of the sexed sorted sperm process is about 90% (Seidel, 2003), we carried out experiments with embryos produced with this type of sperm in an attempt to produce more male embryos. In fact, we obtained 100% presumptive male embryos in this small sample set. The sex determination of bovine embryos using *TSPY*

sequences may be useful in future efficiency studies of sorting sperm.

When we studied embryos conceived with unsorted semen, we chose the morphologically superior embryos, with higher number of cells, which could explain the disproportion of males and females (7:3, respectively). This was observed in the embryos conceived by non-sexed semen, probably due to differences in the *in vitro* development between males and females bovine embryos.

The results of 100% (36/36) of blastomeres (single cells) matched the data from the remaining cells of the same embryo. The mechanism that explains the accuracy of our results is that the *TSPY* is multicopied in the male bovine genome and differs from other Y-specific genes such as the *SRY* gene utilized in some studies, which has only one copy.

Due to the importance of preimplantation sexing and genetic diagnosis, it is necessary to have highly sensitive and specific techniques. On the other hand, they also need to be inexpensive and less time-consuming. This protocol could be used as a screening test of embryo sex in expression experiments using pools of embryos, and to test accuracy of the sexed sorted sperm process or the quality of sexed sorted sperm lots. Another technical option could be real-time PCR, but the equipment required is much more expensive than the one used for a simple nested PCR. The latter can be found in services with low complexity and financial resources. It is a very important advantage, for example, in developing countries.

The present study is the first report in the literature using the *TSPY* gene for bovine embryo sexing of a single cell.

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REFERENCES

Bartmann AK, Ramos ES, Caetano LC, Rios AF, et al. (2004). *TSPY* detection in blood, buccal, and urine cells of patients with 45,X karyotype. *Am. J. Med. Genet. A.* 130A: 320-321.

Bermejo-Alvarez P, Rizos D, Rath D, Lonergan P, et al. (2008). Epigenetic differences between male and female bovine blastocysts produced *in vitro*. *Physiol. Genomics* 32: 264-272.

Chrenek P, Boulanger L, Heyman Y, Uhrin P, et al. (2001). Sexing and multiple genotype analysis from a single cell of bovine embryo. *Theriogenology* 55: 1071-1081.

Hamilton CK, Favetta LA, Di Meo GP, Floriot S, et al. (2009). Copy number variation of testis-specific protein, Y-encoded (*TSPY*) in 14 different breeds of cattle (*Bos taurus*). *Sex Dev.* 3: 205-213.

Jakubiczka S, Schnieders F and Schmidtke J (1993). A bovine homologue of the human *TSPY* gene. *Genomics* 17: 732-735.

Lee JH, Park JH, Lee SH, Park CS, et al. (2004). Sexing using single blastomere derived from IVF bovine embryos by fluorescence *in situ* hybridization (FISH). *Theriogenology* 62: 1452-1458.

Lemos DC, Rios AFL, Lobo RB and Vila RA (2005). Use of the *TSPY* gene for sexing cattle. *Genet. Mol. Biol.* 28: 117-119.

Rios AFL, Lemos DC, Fernandes MB and Andrea MV (2007). Expression of the *CTCF* gene in bovine oocytes and preimplantation embryos. *Genet. Mol. Biol.* 30: 1202-1205.

Seidel GE Jr (2003). Economics of selecting for sex: the most important genetic trait. *Theriogenology* 59: 585-598.

Vogel T, Dechend F, Manz E, Jung C, et al. (1997). Organization and expression of bovine *TSPY*. *Mamm. Genome* 8: 491-496.

Zoheir KM and Allam AA (2010). A rapid method for sexing the bovine embryo. *Anim. Reprod. Sci.* 119: 92-96.

