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Use of the Non-electrophoretic Method to Detect Testis Specific Protein Gene for Sexing in Preimplantation Bovine Embryos*

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ABSTRACT : Testis-specific protein (TSPY) is a Y-specific gene, with up to 200 copy numbers in bulls. In order to make bovine embryo sexing under farm condition more feasible, the possibility of using a non-electrophoretic method to detect the TSPY gene for sexing bovine early embryos was examined. Primers were designed to amplify a portion of the TSPY gene and a common gene as an internal control primer. PCR optimization was carried out using a DNA template from bovine whole blood. Furthermore, embryo samples were diagnosed by this method and the sexing results were contrasted with those of the Loop-Mediated Isothermal Amplification (LAMP) method. The results showed that TSPY was as reliable a sexing method as LAMP. Forty-three morula and blastocyst embryos collected from superovulated donor dairy cattle were sexed by this method, and twenty-one embryos judged to be female embryos were transferred non-surgically to recipients 6 to 8 days after natural estrus. Out of 21 recipients, 9 were pregnant (42.86%) and all delivered female calves. The results showed that the sex predicted by this protocol was 100% accurate. In conclusion, the TSPY gene was a good male specific marker and indicated that a non-electrophoretic method was feasible and accurate to detect the TSPY gene for sexing preimplantation bovine embryos. (**Key Words** : Bovine Embryo, Sex Determination, TSPY Gene, PCR)

INTRODUCTION

PCR techniques for sex determination in the bovine embryo using SRY, ZFY and amelogenin genes have been developed in China (Zeng et al., 1992; Gong et al., 1997; Chen et al., 1999). However, the technique is still relatively difficult to realize in field practice. The native kit has not yet been developed for commercial production and application. Judging from the international sphere, the United States, France, Finland, Japan and other countries have already launched several expensive products (Bredbacka et al.,1995; Hasler et al., 2003), and the cost of directly employing such reagents under our conditions has been identified to be more than 100 Yuan. The success of sex identification technology in bovine embryo depends on the sensitivity and stability. Because the cells from the embryo are relatively small in number, the sensitivity is crucial. Because SRY, ZFY and amelogenin genes are single copies, the same primers used to make more than two domestic or nested PCR amplifications of male-specific genes and detection methods using electrophoresis products were time consuming (Assen et al., 1990; Park et al., 2001) and involved the risk of deoxyribonucleic acid (DNA) contamination of subsequent assays (Bredbacka et al., 1998).

TSPY, the testis-specific protein coded by the Y-chromosome is a Y-specific gene (Affara et al., 1996). TSPY homologues exist in several mammalian species, including humans, horses and cattle (Jakubiczka et al., 1993; Schempp et al., 1995; Vogel et al., 1997). TSPY expression in humans and cattle is apparently restricted to male germ cells and their precursors (spermatogonial cells and spermatocytes), and begins during fetal development. The cellular site of expression suggests a function in spermatogonial proliferation (Vogel et al., 1997). The TSPY gene of cattle has seven exon components, surrounded by six separate introns. TSPY gene copy numbers range from 20 to 60 in men, and reach 200 copy numbers in bulls (Manz et al., 1998). Manz et al. (1998) also reported the use of the TSPY gene for sexing equine transplanted embryos. Pierce et al. (2000) also used successfully the TSPY gene to create a more accurate identification of the sex of human

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Table 1. Primer sequences and annealing temperature

Primer	Primer sequences	Optimum annealing temperature (°C)	Product sizes (bp)
T_1	5'-ACGAAGACGAAAGGTGGC- 3' 5'-CCTGTATGTGAAGGGGTG-3'	53.7	964
T_2	5'-CCCGCACCTTCCAAGTTGTG- 3' 5'-AACCTCCACCTCCTCCACGATG- 3'	64	260
T_3	5'-CCGCCATTACGCCCCCGACTTG–3' 5'-GGGCCGCTGTTCCTGCTCCTCAT–3'	64.8	418
B	5'-TGGAAGCAAAGAACCCCGCT-3' 5'-TCGTGAAAACCGCACACTG-3'	57.5	216

embryos by real-time PCR. At present, there is no research report on the use of the TSPY gene for bovine embryo sexing. This paper is focused on the use of TSPY multi-copy genes to create a more sensitive PCR system which is low-cost, user-friendly and with high sensitivity, suitable for embryo sexing in dairy cattle.

MATERIALS AND METHODS

Materials

Blood samples were obtained from male and female cattle located on the farm of Shandong Academy of Agricultural Sciences. Ten ml of blood was collected by jugular venipuncture into a tube containing sodium citrate as anticoagulant and stored at -20°C until used. λ-DNA and the DL2000 were purchased from Dalian TaKaRa.

Preparation of blood template

The frozen blood was thawed at 37°C; 100 μl of the blood was used to extract high purity DNA by a genomic DNA purification kit (Blood Genome DNA Extraction Kit, TaKaRa) according to the manufacturer's instruction and the concentration of template DNA was estimated with λ DNA (undigested).

Source of embryos and splitting method

Using the conventional superovulation protocol previously outlined by Liu and Huang, (2004), morula and blastocyst stage embryos were non- surgically flushed from Australian Holstein cattle. The embryos were individually placed in the Petri dish with 3-4 drops PBS (200 μl each droplet), and split with a metal blade MN-151 (Narishige, Japan) under the stereomicroscope (SZX7-3121, Olympus, Japan).

Preparation of embryo DNA template

Alkali treatment: The cells were washed three times with PBS and samples in 2 μl of PBS were transferred into 200 μl PCR tubes (Eppendorf PCR tubes) containing 5 μl ALB (50 mM DTT and 200 mM KOH), respectively. The tubes were kept at -80°C before being processed further. The cells were lysed by the addition of 5 μl neutralization liquid (900 mM Tris-HC1, pH 8.3, 300 mM KCl and 200 mM HCl) at 65°C for 10 min. The above solution (5 μl) was added into each reaction tube with male-specific primers and the control reaction tube with the common primers.

Primer design

Male-specific primers T_1 was designed with Primer 5.0 software according to the reported sequence (GenBank accession U75895- TSPY gene *Bos taurus*). TSPY specific primers T_2 and T_3 were designed with DNAstar according to the reported sequence (GenBank accession X74028-*B. taurus* TSPY gene). In addition, the common primers of male and female cattle were designed according to 1.715 microsatellite DNA sequence, with 216 bp PCR product size (Table 1).

PCR

The final reaction volume of 50 μl comprised templates, 10×buffer, 1.5 mM $MgCl_2$, 100 mM dNTP, 1.25 U Taq DNA polymerase (MDBio, Inc., Qingdao, China), 0.1 μM each primer, EB (0 or 1 μl), and ddH_2O. Reaction programs were: 95°C 4min; 94°C 1 min, Tm 1 min, 72°C 30 s and 35 cycles; then kept at 4°C forever. Amplification was performed on a PTC-200 Engine™ thermal cycler (MJ Research Inc., Watertown, MA, USA).

Product testing

PCR products were separated on a 2% horizontal agarose gel. The results were scanned with an Alpha-UVI gel scanning and document imaging system. The male had both male-specific and common bands, while the female had only a common band.

Product testing of non-electrophoresis

Ethidium bromide (EB) was added into the reaction system before PCR amplification. At the end of PCR, the tubes were placed on the UVP (ultraviolet transilluminator) operating at 302 nm. Red fluorescents which appeared in both specific-male and common primer tubes at the same time were identified as male, while only red fluorescent appearing in the common primer tube was identified as female. It was defeated in sampling if there were not red fluorescents in two tubes.

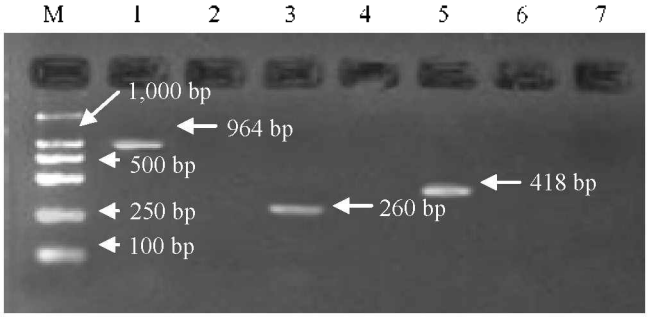

Figure 1. The male blood DNA amplification of PCR using TSPY gene. M: Marker, DL2000 (100, 250, 500, 750, 1,000, 2,000 bp); 1: male sample, primer T_1, 964 bp; 2: female sample, primer T_1; 3: male sample, primer T_2, 260 bp; 4: female sample, primer T_2; 5: male sample,primer T_3, 418 bp; 6: female sample, primer T_3; 7: blank control.

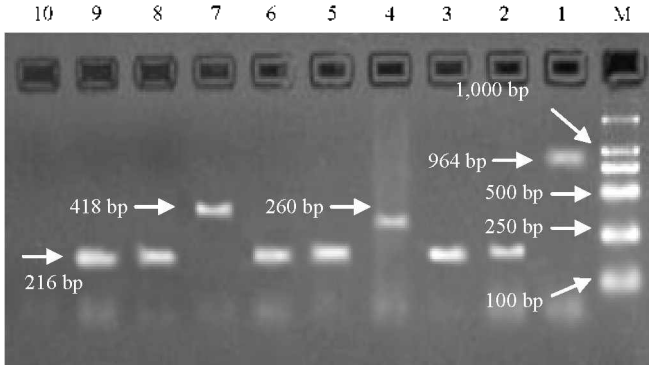

Figure 2. The amplification results (Blood DNA) of PCR of TSPY gene and common gene. M: Marker, DL2000; 1-male sample, primer T_1, 2-male sample, primer B; 3-female sample, primer B; 4-male sample, primer T_2; 5-male sample, primer B; 6-female sample, primer B; 7-male, primer T_3; 8-male, primer B; 9-female, primer B; 10-blank control.

Experiment designs

Experiment 1-Optimization of PCR reaction system and establishment of non-gel electrophoresis method : To establish the non-gel electrophoresis method for sexing bovine embryos, male specific and common primers were selected and the PCR reaction system was optimized using DNA extracted from blood samples of dairy cattle with known sex identity and by simulating the concentration of DNA template derived from sampling embryonic cells.

Experiment 2-Comparison of embryo sexing methods : To evaluate the sex determination methods, the embryo was divided into two parts; one part was treated by alkali to prepare DNA template for the establishment of the non-gel electrophoresis method, while the other part was detected by the (LAMP) method (Huang et al., 2005). Six embryos were primarily detected by the non-gel electrophoresis and LAMP methods.

Experiment 3-Evaluation of sexed embryos following transfer : To assess the efficiency of *in vitro* sexed bovine embryos, forty-three good to excellent quality morula and blastocyst stage (Linder and Wright, 1983), *in vivo* embryos collected from superovulated donor cows 7 days after estrus were determined by the above established method. Approximately 10% of the inner cell mass of 27 morula and trophectoderm of 16 blastocyst stage embryos were split by metal blade, and 21 embryos judged to be female embryos were transferred non-surgically to recipients (Local Luxi Yellow Cattle) 6 to 8 days after natural estrus. Pregnancy diagnosis was performed by rectal palpation 80 days after transfer. The confirmation of *in vitro* sexing was based on the sex of calves at birth.

RESULTS

Experiment 1

Male DNA amplification of PCR using TSPY gene : Figure 1 shows the result of the PCR amplification fragment of the TSPY gene. All samples identified as males showed the expected bands (260,418 and 964 bp) responding to the various TSPY primers, which were not observed in females. This result indicated that three amplification fragments were all male-specific.

Amplification results of PCR of TSPY gene and common gene : Figure 2 shows all male samples had the male specific band and common band, while all the female samples only had the common band, using the TSPY and common primers to amplify the DNA of cow and bull. This result suggested that the common primer with good adaptation for its optimum annealing temperature was different from the three-pair TSPY primers. Indeed, the result indicated that the male specific primers could be amplified with the common primer at the same annealing temperature (53.7, 64 and 64.8°C).

PCR sensitivity of different TSPY gene primers : The sensitivity of the PCR was investigated using different concentrations of male DNA from blood and different TSPY gene primer or common primer. The assay showed all male samples were TSPY-positive and all female samples were TSPY-negative. TSPY-positive results could be detected at the lowest DNA concentration of 10 pg/μl, while the concentration of common primer needed to be above 5 pg/μl. The results indicated that the TSPY gene is male-specific and potentially useful for the sex determination of bovine embryos. The best T_3 TSPY gene primer and the common primer B were paired for the following study.

Non-electrophoresis results of product of PCR at low concentration DNA (Blood) : It is difficulty to analysis the results of gel electrophoresis for the low concentration of DNA (from 10 pg/μl to 60 pg/μl). Therefore, non-electrophoretic detection, namely the direct fluorescence comparison method, was used instead of the gel

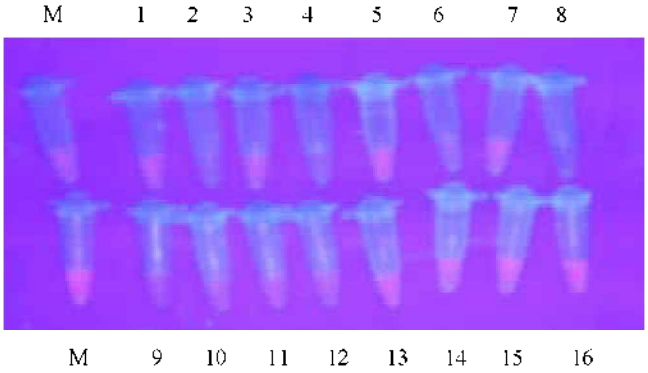

Figure 3. The non-electrophoresis results of product of PCR at low concentration DNA (Blood). M: male control, 1, 3, 5, 7 male, primer T_3; 2, 4, 6, 8 female, primer T_3; 9, 11, 13, 15 male, primer B; 10, 12, 14, 16 female, primer B; 1-8, Concentration of DNA were 10, 10, 20, 20, 40, 40, 60, 60 pg/μl, respectively. 9-16, Concentration of DNA were 10, 10, 20, 20, 40, 40, 60, 60 pg/μl, respectively.

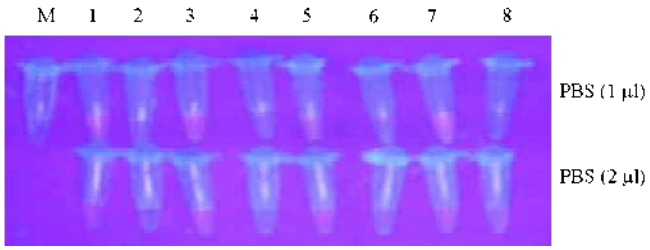

Figure 4. The effect of PBS on non-electrophoretic sex determination efficiency. M: blank control; 1-8, Concentration of DNA (Blood) were 10, 10, 20, 20, 40, 40, 60, 60 pg/μl, respectively. 1, 3, 5, 7 tubes were added with TSPY gene primer and male DNA sample, while 2, 4, 6, 8 tubes were added with TSPY gene primer and female DNA. First lane PBS (1 μl), second lane PBS (2 μl).

electrophoresis method. The result of the non-electrophoresis examination was accurate, which was consistent with the known sex (Figure 3).

Effect of PBS on the result of non-electrophoretic detection : In the embryo sample treatment process, it is possible to introduce inevitably small volume of PBS to the reaction system; therefore, we tested the effect of PBS (1-2 μl) on PCR. The result showed that a small volume of PBS introduced did not affect the reaction system, the mixture of

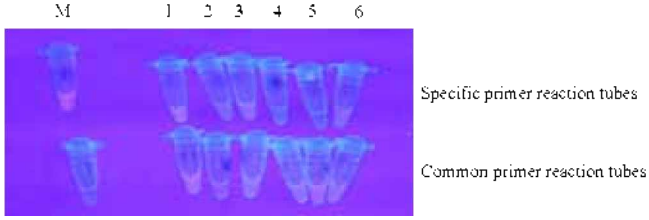

Figure 5. Non-electrophoretic sex determination using specific and common primers. Sample 1-6 were embryos: Lane1 (specific primer reaction tubes): M, male control; 1, 3, 6 tubes with fluorescence; 2, 4, 5 tubes without fluorescence. Lane2 (common primer reaction tubes): M, blank control; 1-6 tubes with fluorescence.

specific primer and common primer was still able to display the high sensitivity (Figure 4).

Experiment 2

Sex determination by non-electrophoresis and LAMP methods : As shown in Figure 5, the result of non-gel electrophoresis examination indicated that embryo samples 1, 3 and 6 were males, while samples 2, 4 and 5 were females. It was consistent with the result of the LAMP method (Table 2).

Experiment 3

Sexing embryo by non-electrophoresis method and embryo transfer : The non-electrophoresis method was employed to determine the sex of *in vivo* derived bovine embryos. Twenty-one embryos judged to be female embryos were transferred non-surgically to recipients 6 to 8 days after natural estrus. The rate of pregnancy was 42.86%. Nine female calves were born. The result indicated that the sex predicted by this protocol was the same as the newborn calves, confirming the accuracy of this sex determination method to be 100%.

DISCUSSION

The PCR technology enhanced the sex determination sensitivity and the accuracy rate in the early embryo. The specificity of gene primer has a direct influence on the accuracy of sex determination in bovine embryos. So, one of the aims of PCR technology used to sex embryo keys is

Table 2. Sex determination results of bovine embryos by non-electrophoresis and LAMP methods

Embryo sample	Non-electrophoresis method		Identified results of non-electrophoresis method	Results of LAMP method
	Specific tube	Common tube		
1	+	+	Male	Male
2	-	+	Female	Female
3	+	+	Male	Male
4	-	+	Female	Female
5	-	+	Female	Female
6	+	+	Male	Male

"+" Mean red fluorescence; "-" Mean without red fluorescence.

to screen male specific primers with the strong specificity and the high sensitivity. At present, much applied research is focused on molecular biology examination methods using PCR technology to amplify the Y chromosome sequence, such as SRY, amelogenin and ZFY genes and repeated sequence (Aasen et al., 1990; Ennis et al., 1994; Utsumi et al., 1994; Virta et al., 2002; Kageyama et al., 2004). A single copy gene was proved to be adequate for sexing, but sometimes failed to amplify from the small amount of DNA template (Levinson et al., 1992; Hochman et al., 1996). Use of repeated Y -specific sequences increased the amount of PCR product, while there was an increase in error risk for the existence of homologous sequences on the X-chromosome (Bardbury et al., 1990). Park et al. (2001) detected the product with a minimum of 5 pg blood DNA template by optimizing consecutive and multiplex PCR. The resulting sexing efficiency was 92.1, 94.3, 96.3 and 100% when 1, 2, 4 and 8 blastomeres were isolated from the bovine embryo, respectively. In experiment 1, the male specific gene primer was designed according to Y chromosome coded testis special protein gene (TSPY), a multi-copy gene. The result showed that the product could be examined with only 10 pg blood DNA template using conventional PCR. Its high sensitivity is possibly related to its high gene copy number. Usually, the DNA content of a one-cell sample embryo is 5pg, which provides the possibility of sexing bovine embryos using the TSPY gene. The result indicated that the TSPY gene was a very good male specific marker, whose primers could satisfy the need of sex determination in bovine embryos with high specificity and sensitivity.

In this study, TSPY-positive results could be detected at the lowest DNA concentration of 10 pg/μl with three pairs of TSPY primers. The amplification stability and quality of PCR product in primer T_3 was the best, and actual time of the PCR process was also the shortest for its highest annealing temperature (64.8°C). Furthermore, the male-specific and common primers could be amplified in the same reaction procedure. Primer T_3 and primer B were paired for the above reasons.

Biopsy and splitting are harmful to the embryo viability and reduce pregnancy rate after sexed embryo transfer (Thibier and Nibart., 1995; Hasler et al., 2002). Since DNA template derived from embryo cells is very low, the conventional electrophoresis method is unable to be performed. Moreover the potential pollutants in the electrophoresis examination process might obstruct the correct result. Recently, several sex determination methods for bovine embryos based on PCR without electrophoresis detection have been developed (Bredbacka et al., 1995; Hasler et al., 2002). Therefore, we have selected the more effective method to fit the low quantity product examination of PCR, namely, EB was inserted in DNA to launch the ultraviolet photoexcitation and create the strong and the weak fluorescence, for visual observation and result analysis in the PCR tube. Because the fluorescence intensity is proportional to the amount of DNA in the tube, this method may achieve equally good results compared with other methods such as electrophoresis examination (Higuchi et al., 1992). Bredbacka et al. (1995) reported that the non-electrophoretic detection method was simple, quick and accurate, without turning on the reaction tube after PCR to reduce the time and possibility of pollution. However, there was a possibility of false negatives from failure in sampling without an internal control. In this study, the common gene primer was designed as an internal control to prevent false negative results. This result showed that it was simple and feasible to detect the TSPY gene by the non-electrophoretic method. This PCR product examination method has been widely applied in dominant DNA marker assistance selection, hybrid (variety) testing, DNA fragment clone domain, and so on.

The preparation of micro-embryo DNA template is another key technology to ensure the accuracy of sex determination in bovine embryos. Several embryonic cell lysis methods have been reported, such as freeze-thawing (Machaty et al., 1992; Chen et al., 2004), heat treatment (Chen et al., 2004), NaOH (Hirayama et al., 2004) and proteinase K (Peura et al., 1991; Hochman et al., 1996; Chrenek and Bulla, 2002; Virta et al., 2002). Hirayama et al. (2004) reported that when DNA of single blastomeres was extracted using heat, NaOH, and proteinase K method, the rates of correct determination of sex were 88.9-94.4%, with no difference among the three methods. However, treatment with proteinase K needed to be deactivated, and easy to deactivate sometimes for long store. In the present study, the result of sexing embryos also showed that the alkali treatment (KOH+DTT) was effective to prepare the micro-embryo DNA.

Many reports showed that the sexing efficiency was 82-96% by the PCR technique (Aasen et al., 1990; Bredbacka et al., 1995; Thibier et al., 1995; Kageyama., 2004). The results from Experiment 2 and Experiment 3 showed that the high embryo sexing accuracy of 100%, was related to the use of a multi-copy gene and a non-electrophoretic method. The rate of pregnancy in Experiment 3 was 42.86%. However, the rate of pregnancy of reported results was 49-62% (Thibier et al., 1995; Shea et al., 1999; Hasler et al., 2002; Hirayama et al., 2004), seemingly higher than that of Experiment 3. This might be affected by the quality of recipient and weather in this experiment. We shall apply further the non-electrophoretic detection method to sex embryos in large numbers.

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