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Sexing Goat Embryos by PCR Amplification of X- and Y- chromosome Specific Sequence of the Amelogenin Gene*

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ABSTRACT : The objective of this study was to develop a simplified, efficient, and accurate protocol for sexing goat embryos. Based on the amelogenin gene located on the conservation region of X- and Y- chromosomes, a pair of primers was utilized and the system of PCR was established to amplify a 262 bp fragment from the X- chromosome in female goats, and a 262 bp fragment from X- chromosome and 202 bp fragment from the Y- chromosome in male goats, respectively. The accuracy and specificity of the primers were assessed using DNA template extracted from goat whole blood sample of known sex. 100% (10/10) concordance was obtained by using the PCR assay. Fifty-one biopsied embryos were transferred into 25 recipient goats on the same day that the embryos were collected and sex of the kid was confirmed after parturition. Eighteen kids of predicted sex were born. The biopsied samples from 51 goat embryos were amplified with 100% efficiency and 94.7% accuracy. In conclusion, our results indicated that PCR sexing protocols based on the amelogenin gene is highly reliable and suitable for sex determination of goats. (**Key Words** : Goat Embryos, Amelogenin Gene, Sexing, PCR)

INTRODUCTION

Goats are important livestock for multiple purposes, such as high quality goat meat and milk (Dhanda et al., 2003), production of valuable recombinant proteins in transgenic goat milk (Pollock et al., 1999) in agriculture production. Recent studies have demonstrated that live goat kids can be produced from in vivo production and in vitro produced goat embryos by embryos transfer (Malader et al., 2007). So, in the goat industry, it is desirable to control the sex ratio of animals.

Several protocols have been established for sexing embryos in farm animals, such as karyotyping (King, 1984), H-Y antigen detection (Andersib, 1987), X-linked enzymatic determination (Monk and Handyside, 1988), and based on the identification of the Y chromosome, such as SRY, ZFY and TSPY genes, include *in situ* hybridization, Southern dot blotting, polymerase chain reaction (PCR), Loop-Mediated Isothermal Amplification (LAMP) (Miller, 1991; Bredbacka and Peippo, 1992; Gutiérrez-Adán et al., 1996; Ng et al., 1996; Solnı et al., 2002; Huang et al., 2006). Among of these methods, PCR-based sexing assays are generally favored, because of the advantages of being relatively simple, rapid, and inexpensive. The first demonstrated sexing of goat embryo with PCR amplified DNA from blood sample was in 1990(Aasen and Medrano, 1990). Subsequently, an accurate, reliable and rapid PCR method had been standardized for accurate sex determination in goats (Rao and Totey, 1992). Leoni et al. (1996) were the first to describe a method for sex determination in goat embryos, using PCR and restriction fragment length polymorphism (RFLP) analysis. They amplified a DNA fragment derived from four to eight cells that had been biopsied from embryos described by Aasen and Medrano (1990). However, the risks would be increased in contamination and misdiagnosis, because of limited amount of DNA in embryo biopsies, cross-species contamination (Aasen and Medrano, 1990; Gutiérrez-Adán et al., 1996), and requirement more time to specific endonuclease digestion for RFLP analysis (Aasen and Medrano, 1990).Therefore, improvement of embryo-based techniques is essential in this species.

The amelogenin gene, which exists on both X- and Y-

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chromosomes (AMELX and AMELY), encodes an important protein in the developing mammalian tooth and enamel matrix that has been conserved during the evolution of vertebrates. Several studies have showed the amelogenin amplification by PCR is a reliable method for sex determination in humans (Sullivan et al., 1993), cattle (Chen et al., 1999), sheep and deer (Pfeiffer and Brening, 2005), goats (Chang et al., 2006; Weikard et al., 2006) as well as in the related species (Weikard et al., 2006). The use of this gene has made the sex determination much less complicated, since only pair of primers is required to amplify the different size fragments of the amelogenin genes (Chen et al., 1999; Weikard et al., 2006). In this study, the possibility of simultaneous amplification of sequences corresponding to both X- and Y- amelogenin gene to establish a reliable, reproducible and efficient PCR-based goat sexing system was tested.

MATERIALS AND METHODS

Reagents

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated. Tissue culture medium 199 (TCM199) was procured from Gibco BRL, USA. Agarose, RNase A, proteinase K, and the PCR reagent were purchased from Promega, Madison, USA. CIDR containing 0.3 g progesterone (EAZI-BREED™ CIDR®; Pharmacia and Upjohn, New Zealand), follicle stimulating hormone (FSH) (Folltropin-V; Bioniche, Belleville, Ontario, Canada), and prostaglandin (Ningbo Hormone Products Co., Ltd., China) was used for synchronization and superovulation of donor and recipient goats.

Preparation of DNA samples from blood

Blood samples were obtained from 10 Boer goats (5 female, 5 male). Genomic DNA was obtained from blood samples by digestion with proteinase K followed by phenol/chloroform extraction and ethanol precipitation (Sambrook and Russell, 2001). In this method, 750 μl of blood was mixed with equal volume phosphate buffered saline (PBS) and centrifuged at 12,000 rpm for 10 min. After discarding the supernatant, 450 μl DNA lysis buffer (10 mM Tris-Cl, pH 8.0, 0.1 M EDTA, pH 8.0, 0.5% SDS (v/v)) was added to the tube containing the cell pellet, followed by added 20 μg/ml RNase A and incubated for 1 h at 37°C. Proteinase K (100 μg/ml) was added to the mixture and incubated overnight at 55°C. Following digestion cooled the solution to room temperature and added an equal volume of phenol equilibrated with Tris-Cl (pH 8.0), mixed gently and centrifuged at 12,000 rpm for 10 min. Then, transferred the viscous aqueous phase to a fresh centrifuge tube, added equal volume of phenol: chloroform (v:v, 1:1), mixed gently and centrifuged at 12,000 rpm for 10 min. After centrifugation, the top aqueous layer was transferred into a clean tube containing absolute ethanol. The precipitated DNA was pelleted down and the supernatant discarded. The DNA pellet was washed gently with 70% ethanol, and air-dried. The DNA was then dissolved in 30 μl of Tris-Ethlenediamire tetracetic acid (TE) buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, and pH 8.0).

Collection of goat embryos

Synchronization of estrus in 29 donor and recipient Boer goats (Parous, aged 2-4 years) was induced by CIDR inserted for 11 days (day 0, CIDR insertion). On the day of CIDR removal, 2 ml prostaglandin (Ningbo Hormone Products Co., Ltd., China) was administered intramuscular. The donors were superovulated with a total dose of 200 mg FSH starting on day 8, with decreasing dose schedule (50, 50, 25, 25, 15, 15, 10, and 10 mg) at 12 h intervals. When the onset of the estrus was detected (1-2 days after CIDR removal), donor were mated with bucks two times daily in the morning and afternoon. Six or seven days after mating, embryos were collected transcervically as described by Holtz et al. (2000) and Suyadi et al. (2000). By transcervical insertion of a flushing catheter (Ch 12, Ruesch, Kernen, Germany, Catalogue No. 220500), each uterine horn was flushed with 20-30 ml of pre-warmed flushing medium (PBS containing 100 IU/ml penicillin, 100 μg/ml streptomycin, 10% fetal bovine serum). Embryos were classified based on morphological characters according to the method of Lindner and Wright (1983). 51 morphologically normal blastocysts from 29 different donors were selected for biopsied.

Embryos biopsy and transfer

Embryos were washed in protein-free TCM199 (containing 100 IU/ml penicillin, 100 μg/ml streptomycin) and biopsied. One embryo at a time was transferred to drop of protein-free TCM199 on a leveled glass plate placed on the warm stage of an inverted microscope (Leica, Germany) and visualized at 100× magnification. Approximately 10% trophoblastic cells were removed from each embryo by cutting with a microrazor blade (Feather Safety Razor Co., Ltd., Osaka, Japan). The excised cells were transferred to a reaction tubes (Hotstart, Molecular Bio-Products, Inc., San Diego, CA) containing 1×PCR buffer. Following biopsy removal, embryos were maintained in holding medium (TCM199 containing 100 IU/ml penicillin, 100 μg/ml streptomycin, 10% FBS) at 39°C under 5% CO_2 in air, until they were transferred into recipients. For each recipient, two or three embryos were aspirated with 20 μl PBS in a glass capillary connected to a 1 ml syringe and were introduced

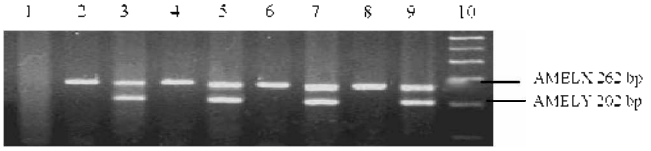

Figure 1. Sexing of goat known gender using PCR assays based on the amelogenin gene. Male blood samples presented 202 bp and 262 bp bands while female blood samples had only 262 bp bands.

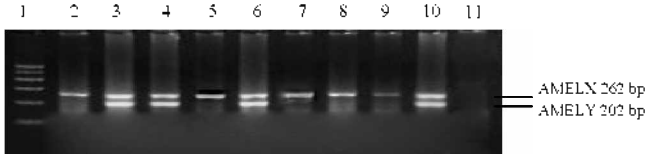

Figure 2. Sexing of goat embryonic cells using PCR assays based on the amelogenin gene

surgically into the top of the uterine horn ipsilateral to the ovary with at least one prominent corpus luteum of healthy parous Boer goat.

Preparation of DNA samples from embryos

The DNA was isolated from the embryos by the single step method described by Taneja et al. (1998) and Saravanan et al. (2002) in 1×PCR buffer (10 mM Tris-Cl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$) containing proteinase K (150 μg/ml) and incubated for 30 min at 37°C. Then proteinase K was inactivated by incubating at 97°C for 10 min. The tubes were kept frozen at -20°C until sexing was carried out.

PCR amplification

Sexing was performed according to the method developed for goat embryos by Ennis and Gallagher (1994) and Pfeiffier and Brenig (2005) with minor modifications. The PCR primers were 5'-cagccaaacctccctctgc-3' (SE47) and 5'-cccgcttggtcttgtctgttgc-3' (SE48). The optimum PCR reaction condition was determined by a preliminary experiment with goat blood DNA (data not shown). Amplification of amelogenin specific primers produced 202 bp and 262 bp fragments. The biopsied embryos were transferred into 25 estrus synchronized does, two embryos per recipient, except one recipient was transferred three embryos.

The PCR reaction was performed in a total volume of 20 μl containing 10×PCR buffer 2 μl, 25 mM $MgCl_2$ 1.2 μl, 10 mM dNTPs 0.4 μl, 0.5 U of Taq DNA polymerase and 20 pmol each primer 0.5 μl. PCR was programmed using a PTC-100 Thermal Cycler(MJ Research, Watertown, MA) for 94°C for 3 min, followed by 35 cycles of one denaturation step at 94°C for 35 sec, primer annealing at 61°C for 50 sec and primer extension at 72°C for 35 sec. At last cycle, the samples were incubated at 72°C for 7 min.

Amplification products (10 μl) were analyzed on 2% agarose gel stained with ethidium bromide and evaluated under ultraviolet light.

RESULTS

Sexing using goat blood sample

PCR amplification of amelogenin gene resulted in a 262 bp and 202 bp fragment corresponding to the goat blood genomic DNA. As shown in Figure 1, male samples presented both bands while female samples had only 262 bp band. Negative samples showed no bands. For confirming the accuracy of the PCR, 10 blood samples (5 females and 5 males) were detected. All sex determination by the PCR was in agreement with the actual sexes of the goats from which blood samples were obtained, indicating that the sexing method based PCR amplified amelogenin gene was 100% reproducible and reliable.

Sexing of goat embryos

A total of 51 goat embryos were used to study the efficiency and accuracy of the method. The 51 embryos were transferred into 25 recipient goats. Five weeks after the transfer of the sexed blastocysts, pregnancy rate of the recipient of does was confirmed. Of the 19 kids born to date 18 were correctly sexed (Table 1). The misidentified embryo was a male diagnosed as a female by PCR. Figure 2 showed the parts results of PCR sexing of the embryos after agarose gel electrophoresis. The samples showing 262 bp and 202 bp fragment were considered to be male embryos, while those showing only 202 bp fragment were considered to be female embryos. At the same time, blood DNA samples of known sex were used as positive control and sterile water was used as negative control.

DISCUSSION

PCR is a rapid, easy procedure for large scale sexing, and primers derived from many Y-specific sequences have

Table 1. Efficiency and accuracy of PCR for sexing of goat embryos

No. of transferred embryos	No. of recipients	No. of kids			
		Born (%)	Male	Female	Accuracy (%)
51	25 24 (2embryos) 1 (3embryos)	19/51 (37.3%)	8	11	18/19 (94.7%)

been used to screen blood, meat and blastomere samples (Zeleny et al., 2002; Alves et al., 2003). Amplification of the amelogenin gene sequence was sufficient to determine the gender and it had been done successfully in bovine embryos and ovine embryos (Pfeiffer and Brening, 2005) sexing studies. Using X- amelogenin gene as an internal control did not interfere with amplification of the Y-specific sequence even 1 ng DNA (Phua et al., 2003).

The objective of this study was to develop a goat sexing assay that was accurate, sensitive, and relatively fast. The most common approach in sexing embryos involves the co amplification of the Y-chromosome specific sequence containing the Y-linked genes (SRY) and an autosomal sequence that acts as a control for the presence of DNA (Mara et al., 2004). In the present study, we employed primers derived from a sequence for X- and Y- specific amelogenin, and verified the accuracy of the assay by evaluating genomic DNA from 5 females and 5 males. The overall amplification products obtained showed 100% accuracy. This result was comparable to those reported by other authors (Ennis et al., 1994; Pfeiffer et al., 2005; Weikard et al., 2006) with the same primers. This assay provides a rapid and sensitive method for sexing, because of the presence of the X- chromosome band. Moreover, it can be carried out in a regular laboratory or under farm conditions within 4 h. This is especially important for the future application of the protocol to caprine embryos sexing.

It is well known that sex and satellite chromosome specific sequences are highly conserved in the Bovidae family during evolution, allowing the use of heterologous PCR primer pairs in closely related species (Moore et al., 1991; Mara et al., 2004). Several protocols made use of bovine Y-chromosome sequence derived primers on sheep blood cell DNA, trophoblastic cells to detect the sex of sheep embryos (Rao et al., 1992; Mara et al., 2004). The conserved status of the amelogenin gene among vertebrates indicates the possibility to use the test in cattle, sheep, red deer, and other mammal species (Pfeiffer and Brening, 2005). Our protocol used bovine DNA derived primers on goat blood genomic DNA and trophoblastic cells, suggesting it as a potential method to detect the sex of goat embryos.

The method described in this study consistently produced fixed banding patterns in male and female DNA samples extracted from blood of known phenotypic sex. The negative control resulted in no amplification, thus foreign DNA contamination was excluded. Moreover, our study showed there was no contamination from human DNA during the laboratory analysis. These were minimized the risk of contamination, and insured the accuracy of sexing.

The method of biopsy and sex determination of goat embryos that we used can accurately and efficiently predict the sex of embryos before transfer. The microblade used to biopsy the embryo is easy to use and has proven to be effective with bovine and sheep embryos (Herr and Reed, 1991; Kochhar et al., 2000). Quality of the embryo and the size of the biopsy can influence the outcome of the procedure (Ju et al., 2001). We used morphologically normal blastocysts and obtained a 37.3% kidding rate after transfer. These results are comparable with *in vitro* produced ovine embryos, biopsied and sexed by similar technique (Mara et al., 2004).

In our study, one of male was misdiagnosed as female. It may be due to a sample, exhibiting a weak band thus presumed to be a female (Mara et al., 2004). In addition, spermatozoa are frequently attached to the zona pellucida in goats (El-Gayar and Holtz, 2005), thus interfering with embryo sexing (Schmoll and Schellander, 1996; Verlinsky et al., 1996; El-Gayar and Holtz, 2005). However, we were not able to determine precisely the reason for error in diagnosis. Furthermore, an accurate appraisal of reliability of the sexing technique employed in this investigation will have to be based on a substantially larger number of transfers.

In conclusion, with the X- and Y- chromosome specific sequence of the amelogenin gene, we improved the conventional PCR-based goat embryo sexing method. With a single primer set, two PCR products, a male-specific PCR product and a PCR product present in both sexes, can be detected simultaneously. The method can be used in goat breeding programs to manipulate sex ratios of offspring.

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