



Molecular sexing of IVF and *in vivo* derived embryonic cell colony in goat

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ABSTRACT

The present study was designed to standardize the molecular sexing of caprine embryos from few blastomere of IVF- and *in vivo*- derived embryonic cell colony in one step by real time PCR. The cumulus oocyte complexes (COC) were isolated, evaluated, graded and matured in maturation media and after maturation, the oocytes were incubated with 50µl capacitated spermatozoa of proven buck from experimental shed of this institute for IVF embryo production. Surgical embryo collection was done from 10 goats as per standard protocol and mated with a buck of proven fertility. Embryonic stem cell culture method was used for generation of embryonic cell colony from IVF- and *in vivo*- derived embryos of 8–16 cells and morula. Genomic DNA was isolated from blood sample for standardization of SRY gene expression. Genomic DNA was also isolated from 8–16 cell and morula stage of *in vivo* and IVF derived embryonic cell colony. Sexing of IVF- and *in vivo*- derived embryonic cell colony was done with SRY gene expression using real time PCR. The sex ratio of male: female embryos were 1: 1.5 and 1: 1 in 8–16 cell and morula stage *in vivo*- embryo derived embryonic cell colony respectively. For IVF embryos the sex ratio of male: female was 1: 1.25 and 1.1: 1 in 8–16 cell and morula stage of derived embryonic cell colony respectively. The overall sex ratio of male: female embryos were 1: 1.18 in our study.

Key words: Embryo sexing, Goat, Embryonic cell colony, SRY

Goat is reared for multiple purposes and it is desirable in the goat industry to control the sex ratio of animals. Embryo sex predetermination can help in building up and drawing the outlines of a certain management strategy within the livestock.

Karyotyping (King 1984), H-Y antigen detection (Andersib 1987), X-linked enzymatic determination (Monk and Handyside 1988) and techniques based on identification of Y chromosome, such as SRY, ZFY and TSPY genes, polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP) (Miller 1991, Bredbacka and Peippo 1992, Sohn *et al.* 2002, Huang *et al.* 2007) are used for sexing embryos in farm animals. Among these techniques PCR-based sexing is the best because of being relatively simple, rapid, and inexpensive. The first goat embryo sexing was demonstrated with PCR amplified DNA from blood sample (Aasen and Medrano 1990, Rao and Totey 1992). Leoni *et al.* (1996) described a method for sex determination in goat

embryos using PCR and restriction fragment length polymorphism (RFLP) analysis.

Over recent years, different methods have been proposed for sex determination of embryonic blastomeres, but most of these methods are multi-step, lacking the full accuracy, sensitivity and may require a technical skill. As improvement of embryo-based techniques is essential in this species, the present study was designed to standardize the molecular sexing of caprine embryos from few blastomere of IVF- and *in vivo*-derived embryonic cell colony in one step by real time PCR.

MATERIALS AND METHODS

Ethics: All the experiments were approved by the Indian Veterinary Research Institute, Izatnagar, Bareilly, U.P. India ethics committee.

IVF embryos production: Goat ovaries were collected from the local slaughter house. The cumulus oocyte complexes (COC) were isolated, evaluated, graded and matured in maturation media (TCM-199 with HEPES media, 10% FBS, 10% goat follicular fluid, 0.5µg/ml FSH, 10µg/ml LH, 0.5µg/ml estradiol). After maturation, oocytes were washed several times with fertilization TALP media for removing expanded cumulus cell mass, and transferred to

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50µl drop of fertilization TALP media (Totey *et al.* 1996); 50µl capacitated spermatozoa were added to this. Fresh semen was collected from proven buck of experimental shed of this institute. After 18 h of sperm co-incubation with oocyte, the presumptive zygotes were washed several times in mSOF (Walker *et al.* 1996) supplemented with 0.8% BSA and essential and non-essential amino acids and cultured in 100µl of same media in CO₂ incubator at 37°C, 5% CO₂ and 95% relative humidity until assessment to determine cleavage rates. For embryo development cleaved oocytes were subsequently transferred in fresh drop of mSOF and kept in CO₂ incubator.

Surgical collection of embryo: For superovulation healthy female goats, 2 to 2.5 years old, were selected and monitored for cyclicity at least for 3 consecutive cycles. Pluset @ 20 IU per goat was administered intramuscularly in a constant dose schedule. Treated goats were checked for estrus and mated with a buck of proven fertility of experimental shed of this Institute. Surgical embryo collection was done from 10 goats as per standard protocol (Agrawal *et al.* 1982).

Culture of embryonic cells isolated from early stage embryos: The IVF- and *in vivo*-derived embryos of 8–16 cells and morula were zona lysed with proteinase-K (0.02%) and clumped blastomere were washed in 4–5 drops of stem cell culture media [Knockout DMEM supplemented with 10% FBS, LIF (40ng/ml), ITS (0.3%), NEAA (0.5%), SCF (20ng/ml), bFGF (4ng/ml), IGF-1 (100ng/ml)]. After washing, the clumped blastomere were cultured in wells on mitomycin C inactivated goat fetal fibroblast monolayer at 37°C, 5% CO₂ and 90% relative humidity in a CO₂ incubator and the formation of cell colonies was observed. Embryonic cell colonies were carefully eluted and washed 6–7 times to remove any fibroblast cells as fibroblast cell are large in size compared to individual blastomere. A small number of cells from the colony developed from individual IVF and *in vivo* derived embryos of 8–16 and morula stage embryos were used for genomic DNA isolation for sexing.

Genomic DNA isolation from blood/embryonic cell colony: Genomic DNA was isolated from blood sample for standardization of SRY gene expression. Genomic DNA was also isolated from 8–16 cell and morula stage of *in vivo*- and IVF-derived embryonic cell colony by genomic DNA mini kit.

Sexing of blastomere colony: A positive sample from male goat was also run in parallel. The PCR condition was an initial denaturation step at 95°C for 3 min followed by 40 cycles of 95°C for 5 sec, annealing for 15 sec. The forward sequence of SRY gene was F5'TGAACGAAGACGAAA GGTGGCTCT3' and reverse sequence was R5'ATCAGGGACTGTGAGCGGCATAAT3'. The annealing temperature was 60°C and product size was 342bp and gene bank accession no. of is Z30646.1

The data were analyzed by standard statistical method (Snedecor and Cochran 1989).

RESULTS AND DISCUSSION

PCR amplification of SRY gene resulted in a 342 bp fragment corresponding to the goat blood genomic DNA. Male samples showed band and female samples not showed bands (Fig.1). For confirming the accuracy of the PCR, 8 blood samples (4 females and 4 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 SRY gene was 100% reproducible and reliable (Fig. 1). The overall amplification products obtained showed 100% accuracy. This result was comparable to those reported by others (Ennis *et al.* 1994, Pfeiffer *et al.* 2005, Weikard *et al.* 2006, Chen *et al.* 2007).

Sexing of IVF and *in vivo* derived embryonic cell colony was done with SRY gene expression using PCR (Fig. 2). The colony which expressed SRY gene was classified as male colony (Figs. 1, 2). The sex ratio of male: female embryos were 1: 1.5 and 1: 1 in 8–16 cell and morula stage *in vivo* embryo derived embryonic cell colony respectively. For IVF embryos, the sex ratio of male: female was 1: 1.25 and 1.1: 1 in 8–16 cell and morula stage of derived embryonic cell colony respectively. The overall sex ratio of male: female embryos was 1: 1.18 in our study.

Several approaches were used to determine the sex of embryos. At present PCR seems to be the most effective and sensitive method to determine the embryonic sex of domestic animals. Since DNA sequences in single cells can be studied using PCR, sex of livestock embryos fertilized *in vitro*, can

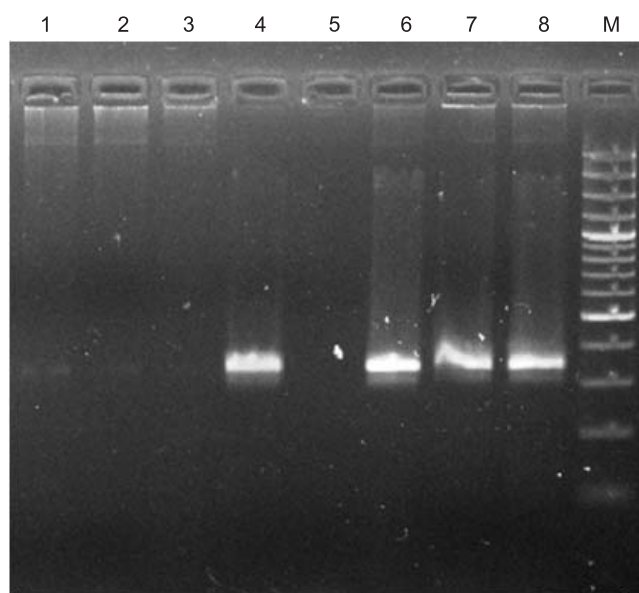


Fig. 1. Standardization of SRY gene expression in goat blood (100 bp DNA Ladder marker). (Lane 1 – 3 and 5 were female genomic DNA and Lane 4, 6–8 were male genomic DNA isolated from blood).

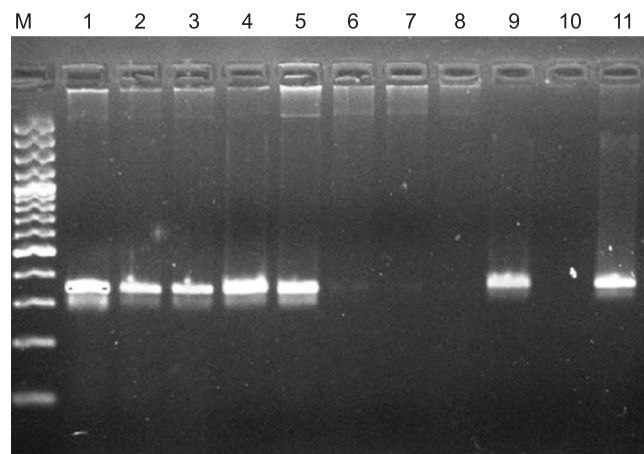


Fig. 2. Sexing of embryonic cell colony with SRY gene expression (100 bp DNA Ladder marker). (Lane 1 – 5, 9 and 11 were female genomic DNA and lane 6–8 and 10 were female genomic DNA isolated from blastomeres of embryonic cell colony).

be determined before implantation. In comparison with earlier method for sex determination, PCR offers the invaluable advantage of being so fast.

We found consistently fixed banding patterns in male DNA samples extracted from blood of known phenotypic sex. The negative control resulted in no amplification, thus foreign DNA contamination was excluded. The method of sex determination of goat embryos that we used can accurately and efficiently predict the sex of embryos.

In conclusion, with the Y- chromosome specific sequence of the SRY gene, we improved the conventional PCR-based goat embryo sexing method. The method can be used in goat breeding programs to manipulate sex ratios of offspring.

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