Sexing of Sheep Embryos Produced *In vitro* by Polymerase Chain Reaction and Sex-specific Polymorphism

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ABSTRACT : The accuracy of Polymerase chain reaction (PCR) assay in sexing of sheep embryos was assessed in this study. A total of 174 ovine embryos produced in vitro at different stages of development (2, 4-8 cell stages, morula and blastocyst) were sexed. The universal primers (P1-5EZ and P2-3EZ) used in this assay amplified ZFY/ZFX-specific sequences and yielded a 445 bp fragment in both sexes. Restriction enzyme analysis of ZFY/ZFX-amplified fragments with Sac I exhibited polymorphism between sexes, three and two fragments in males and in females, respectively. For verification of accuracy, blood samples of known sex were utilized as positive controls in each test. The mean percentages of sex identification by this method at 2 cell, 4-8 cell, morula and blastocyst were 73.00±5.72, 89.77±3.79, 83.33±8.08 and 79.6±9.09, respectively with the over all male to female ratio of 1:0.87. It is concluded that the ZFY/ZFX based method is highly reliable for the sexing of sheep embryos. *(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 5 : 650-654)*

Key Words : Sheep, Embryo Sexing, PCR, Polymorphism, Sex Ratio

INTRODUCTION

Sheep, with their multifaceted utility, play an important role in the agrarian economy. In the last two decades, rapid progress has been made in the development of new technologies in ovine reproduction, of which, embryo transfer plays an increasingly important role in genetic improvement (Boutonnet, 1999; Crawford, 1999). The success of embryo transfer technology can still be improved if embryos of the desired sex are made available at a reasonable cost, since the demand for sexed embryos is strong and ever rising. Several approaches, including karyotyping, immunological detection of male specific H-Y antigen and assay of sex linked enzymes have been used to determine the sex of sheep embryos. However, they failed to gain much popularity due to lack of either accuracy or speed (Betteridge, 1984; Innis and Gelfand, 1990; Bourhis et al., 1998)

Recently, embryo sexing by polymerase chain reaction (PCR) and restriction enzyme (RE) analysis have gained importance and offer an accurate and fast method to determine the sex of bovine (Ennis and Gallagher, 1994), porcine (Fajfar-Whetstone et al., 1993) and equine (Peippo et al., 1995) preimplantation embryos.

This method is usually based on the presence or absence of Y-specific amplification (Herr et al., 1990; Fajfar-Whetstone et al., 1993; Ennis et al., 1999). Another approach is to amplify the sequences in the ZFY (Zinc finger protein Y) locus, which is a single copy gene, located on the Y chromosome and its chromosome homologue, ZFX (Pollevick et al., 1992; Garcio-Muro et al., 1997). In this study we assessed the efficiency of PCR and RE analysis in sexing of ovine embryos at different stages of development.

MATERIALS AND METHODS

Chemicals

All the chemicals were of analytical grade and purchased from Sigma Chemical Co. St. Louis, MO, USA. Agarose (ultrapure), Brinster medium, Heat inactivated fetal calf serum (FCS), Proteinase-K and 100 base pair DNA ladder were procured from Gibco BRL, USA. The PCR core reagent kit was obtained from Finnzymes, Finland and restriction enzymes were purchased from Bangalore Genei Pvt. LTD, India.

Embryos

Sheep ovaries (n=414) were obtained from the Corporation slaughter house, Chennai, Tamil Nadu and transported to the laboratory in normal saline (37°C) containing Gentamicin (80 mg/litre). The oocytes were retrieved by slicing the ovaries (Pawshe et al., 1994). Embryos were produced by in vitro fertilization according to the method of Totey et al. (1993) with few modifications. Briefly, the good quality oocytes were placed in maturation medium (20 oocytes/drop) and cultured for 24 h at 37°C in a CO_2 incubator with 5% CO_2 in air and 90% relative humidity. The maturation droplets were prepared by placing 100 μl of Tissue culture media (TCM) 199 containing 10% serum, 10.5 ng/ml FSH, 5 μg/ml LH and 1 mg/ml 17 β E_2

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Table 1. Composition of media

Composition of Maturation media		Composition of fertilization media	
Particulars	Quantity	Particulars	Quantity
TCM-199	9 ml	TL base (Modified tyrode's lactate solution)	10 ml
Heat inactivated FCS (10%)	1 ml	Sodium pyruvate	1.1 mg
Sodium Pyruvate (0.2mM)	100 µl	Sodium bicarbonate	20 mg
Ovine FSH	0.5 µg/ml	BSA (fatty acid free)	60 mg
Ovine LH	5 µg/ml	Heparin (0.1 mg/ml)	100 µl
L-Glutamine (0.1%)	60 µl		
Gentamicin (50 mg/ml)	10 µl		
17 beta-oestradiol	1mg/ml		

Table2. Sequence of primers used for amplification assay

Primer	Sequence	Sequence length
Forward-5' (P1-5EZ)	5'-ATAAT CACAT GGAGA GCCAC AAGCT-3'	25 mers
Reverse-3' (P2-3EZ)	5'-GCACT TCTTT GGTAT CTGAG AAAGT-3'	25 mers

in a 60×15 mm petridish. The composition of maturation and fertilization media was given in Table 1. Oocyte maturation was assessed after 20-22 h (Fry et al., 1997). Sperms obtained from the cauda epididymis of matured testes were subjected to the gradient (Percoll 45% and 90%) centrifugation for the collection of live spermatozoa. Five µl of sperm suspension (2×10^6 million/ml) was inseminated in vitro into the fertilization droplets (50 µl) containing heparin (0.1 mg/ml) and allowed to equilibrate for 15 min before placing matured oocytes into the droplets (Fukui et al., 1988).

After 24 h co-incubation, the cleaved oocytes were transferred into 50 µl of Brinster medium containing 20% serum and cultured for 2 more days.

DNA extraction from Embryos

The cultured embryos (in different cell stages) were washed twice in culture medium and three times in KCl medium with 2 g/L bovine serum albumin. DNA was isolated from the embryos by the single step method described by Taneja et al. (1998) in a 1×PCR buffer (10 mM Tris HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01% gelatin) containing Proteinase-K (150 µg/ml) and incubated for 1 h at 37°C. Then Proteinase-K was inactivated by incubating at 99°C for 10 min. The tubes were kept frozen at -20°C until sexing was carried out.

DNA extraction from blood

Genomic DNA samples isolated from the blood of known phenotypic sex were used as male and female controls for the verification of accuracy, sensitivity and specificty of PCR in sexing the embryos. Five ml of blood was transferred to three volumes of cold RBC lysis buffer (Ammonium chloride 150 mM, potassium chloride 10 mM and EDTA 0.1 mM) and centrifuged at 4,000 rpm for 10 min to pellet white blood cells. The cell pellet was treated with 25 µl of Proteinase-K, 0.25 ml of 0.5 M EDTA (pH 8.0) and 0.25 ml of 20% sodium dodecyl sulphate (SDS) for a minimum period of 5 h (Montgomery and Sise, 1990). Following digestion, 4.3 ml of saturated sodium chloride (NaCl) solution was added, vortexed vigorously and centrifuged at 5,000 rpm for 15 min at room temperature. The aqueous layer of chloroform: isoamyl alcohol (24:1) extraction was precipitated with two volumes of 95% cold ethanol. The precipitated DNA was resuspended in 400 µl of TE buffer (pH 8.0). The yield and purity of DNA samples were estimated by spectrophotometry.

PCR amplification

The PCR reactions were carried out in 50 µl of PCR buffer (10 mM Tris HCl, pH 8.3, 50 mM KCl and 0.1% TritonX-100), $MgCl_2$ (1.5 mM), four dNTPs (each 200 µM), Taq DNA polymerase (2 units) and oligonucleotide primers (P1 and P2, each 40 pM; sequence listed in Table 2) with 15 µl of embryonic lysate. The mixture was overlaid with 30 µl of mineral oil.

The PCR amplification was carried out using a MJ research thermal cycler with the following amplification procedure: An initial denaturation for 5 min at 95°C was followed by 40 cycles of denaturation (for 60 sec at 94°C), primer annealing (for 60 sec at 56°C) and strand synthesis (for 120 sec at 72°C), and in the last cycle, the samples were held at 72°C for an additional 5 min. The amplification was confirmed by agarose gel electrophoresis (1.2%) stained with 0.5 µg/ml ethidium bromide.

Restriction enzyme analysis

PCR products (15 µl each) were subjected to digestion at 37°C for 3 h with 10 units of Hind III, 10 units of Kpn I, 10 units of Pst I, 20 units of Sac I and 10 units of Bam HI restriction enzymes in the presence of 1×BSA (2 mg), and then inactivated at 70°C for 10 min. The Restriction fragment length polymorphism (RFLP) was then analyzed using 2.5% agarose gel electrophoresis and visualized under UV light.

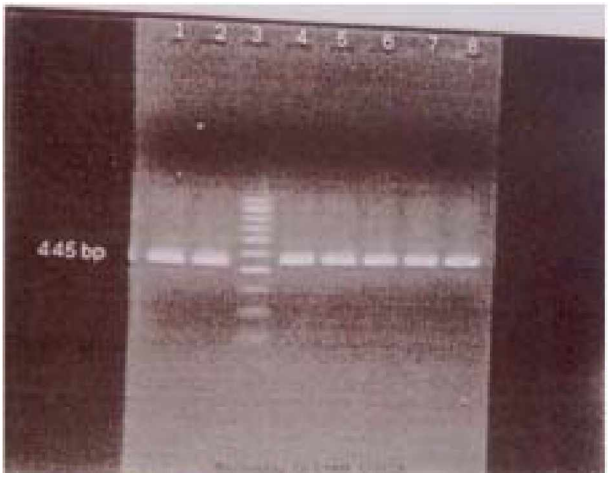

figure 1. Amplification of ZFY/ZFX-specific sequences using universal primers
Lane 1. Male control (blood)
Lane 2. Female control (blood)
Lane 3. 100 bp ladder (marker)
Lanes 4, 5, 6, 7 and 8. Embryo samples

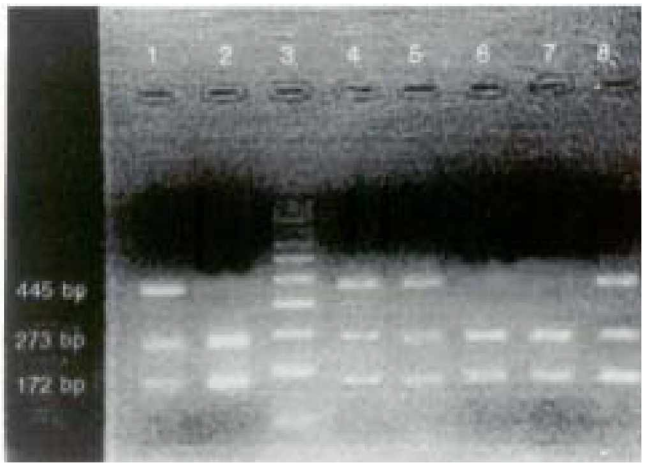

figure 2. Restriction patterns from SacI digested ZFY/ZFX-PCR products of sheep embryos
Lane 1. Male control (blood)
Lane 2. Female control (blood)
Lane 3. 100 bp ladder (marker)
Sexed embryos: Female lanes 4, 5 and 8.
Male lanes 6 and 7.

Data analysis

The mean percentage and angle values corresponding to percentages, of embryos were calculated as per the procedure of Snedecor and Cochran (1994). The sex ratio was identified at different cell stages of the study.

RESULTS

By choosing universal primers from sequences that are highly conserved in the X and Y chromosomes, sex-specific sequences were successfully amplified in embryonic lysates. The embryos subjected to PCR with universal primers showed uniform banding patterns (445 bp) irrespective of sex (Figure 1) (Aasen and Medrano, 1990). The sex was distinguished after digestion with Sac I, due to the presence of a single cut site (GAGCTC) on the sheep X chromosome which is absent on the Y-chromosome. Therefore, the ZFY fragment remained uncut while the ZFX homologue (which is present in both male and female embryos) was digested to give fragments of 172 and 273 bp (Figure 2). The remaining enzymes were not given any restriction pattern between the sexes.

The arc-sin root percentage values of embryos that could be sexed by this method at 2-cell, 4-8 cell, morula and blastocyst stages were 58.69, 71.37, 65.88 and 62.87, respectively. The overall accuracy of sexing was 83.3%. Sex determination was effective in all the four stages of embryo development. Statistical analysis revealed that sexing efficiency was not significantly differed in all the four stages.

DISCUSSION

Several approaches have been 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 be determined before implantation. In comparison with earlier method for sex determination, PCR offers the invaluable advantage of being so fast. The sexing of a large series of embryos could be accomplished in less than 5 hr. We have developed an efficient method of embryo sexing for preimplantation sheep embryos, which greatly enabled us to accomplish sex diagnosing of these embryos from 2-cell stage to blastocysts by amplification of X and Y chromosome sequences by PCR and simultaneous restriction fragment length polymorphism (RFLP). In the present study, universal primers were chosen from sequences that are conserved between human ZFY and ZFX gene and the mouse zfy-1 and zfy-2 genes. This locus is present on the Y chromosome of all placental sites (Leung et al., 1990; Matthews and Reed, 1991).

This method presented a PCR protocol for sexing the ovine embryos of Indian descriptive (Madras Red) and non-descriptive breeds without the need for autosomal control primers. Both ZFY and ZFX genes are phylogenetically conserved, so presence of restriction enzyme recognition sequence on this locus confers a polymorphism between the sexes. Amplification of ZFY and ZFX loci by PCR followed by detection of polymorphism between these amplified loci by RFLP (Matthews and Reed, 1991) have been used successfully in sex determination of many

Table 3. Number and mean percentage of embryos sexed, and sex ratio observed in PCR-RFLP

Cell stage	2 cell	4-8 cell	Morula	Blastocyst	Total
Total No.	82	158	60	48	348
Male	56	74	18	38	186
Female	26	84	42	10	162
Male: Female	1:0.46	1:1.14	1:2.33	1:0.26	1:0.87
Mean percentage	73.00±5.72	89.77±3.79	83.33±8.08	79.16±9.09	-

mammalian species. The method described in this study consistently produced fixed banding patterns in male and female DNA samples extracted from blood of known phenotypic sex. Furthermore, in the analysis of embryonic cells, no misidentifications were observed with in parallel samples of each embryo. These findings are in consonance with those of Aasen and Medrano (1990). The overall sexing efficiency in the present study was 83.3% and it is comparatively lower than that reported by Taneja et al. (1998). They reported that the total amplification success, with BRY1 primers, was 97% after the lysis of samples. With BRY1 primers the absence of bands may not always confer female sex. The increase of convenience and speed of the protocol were compatible with high efficiency and accuracy of sex diagnosis. The distinction was although dependent on species-specific restriction enzymes, that is, the similarity of ZFY and ZFX loci in all mammals makes it possible to use the same protocol for different species with the only difference being the RE used to detect polymorphism between the ZFY and ZFX loci.

The efficiency, sensitivity and reproducibility of the PCR assay depends on several parameters such as the amount of embryonic lysate (template DNA), inactivation of Proteinase-K, primer concentration, $MgCl_2$ concentration and the number of cycles of PCR (Saiki et al., 1998). In this study better reproducibility of the PCR was observed with 1.5 mM $MgCl_2$ and there was no amplification of the embryonic DNA when $MgCl_2$ was used at concentrations of more than 2.5 mM. This is contradictory to the findings of Carvalho et al. (1996), who used a $MgCl_2$ concentration of 2.5 mM for the sexing of bovine embryos.

The accuracy of sexing at different stages of embryo development is shown in Table 3. There was no significant difference in sexing accuracy among all the four stages of development. The DNA content in the two-cell stage may be sufficient for the quantitative amplification by PCR. Hence identification of the sex of ovine embryos at two-cell stage itself may alleviate the need for the culture of embryos up to morulla or blastocyst.

Embryos were produced in vitro from oocytes and spermatozoa collected from ovaries and testes of sheep from an abattoir, so as a preliminary study in India whole embryos were involved in the sex identification assay. But the embryo biopsy and followed by its survival assessment will indeed fulfill the basic need of sex determination and its incorporation with embryo transfer technology, will finally ensure the creation of sizeable population for commercial breeding and production industry in near future.

In conclusion, amplification of ZFY and ZFX loci by PCR followed by digestion with Sac I offers a rapid and accurate method of sex diagnosis of sheep embryos.

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