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Predetermination of Sex in Bovine Embryos by PCR†

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PCR에 의한 소 초기배 성의 급속판정†

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요 약

초기배의 성판정은 대상가축의 성을 선발하는 수단으로써 가축의 육종 및 번식에 있어 가치가 매우 높다. 체세포, 체외수정 또는 처녀발생 초기배의 성을 결정하기 위해 capillary polymerase chain reaction (PCR)을 이용하였으며 성판정에 이용되는 상실배 또는 배반포는 체외수정과 그 후의 난관상피세포와의 공배양에 의해 생산되었다. 초기배의 genomic DNA는 0.2 μg / μL proteinase K를 함유하고 있는 PCR lysis buffer에 하나의 초기배를 부유하여 50℃에서 1시간동안 배양한 후 95℃에서 10분간 효소를 비활성화시킴으로써 준비되었다. 웅성 초기배에서는 두개의 증폭산물(웅성특이 및 소특이)이 생산되는 반면 자성과 처녀발생 초기배에서는 단 하나의 증폭산물(소특이)만이 생산되었다. 이 기법에 의한 성판정 결과 초기배의 성비는 예상되는 1:1의 성비와 유의적인 차이가 없었다. 잔여 난구세포 또는 투명대에 결합된 정자 등으로 인한 잘못된 성판정이 종종 발생하는데, 이는 citrate 처리후 투명대를 완전히 제거함으로써 false positive 또는 negative 결과를 극복할 수 있었다. 이상과 같은 결과는 체외생산 소 초기배의 신속하고(2시간 증폭) 정확한 성판정의 가능성과 초기배 이외의 세포로부터의 오염이 확립된 수세방법에 의해 효과적으로 배제될 수 있음을 제시하였다.

INTRODUCTION

Predetermination of sex in early embryos is of great value in the industry of animal production since it provides a means to select animal sex of interest. Numerous studies on the sex control have been carried out to predetermine the sex of preimplantation embryos using male- or Y chromosome-specific repetitive sequences(Bradbury *et al.*, 1990; Handyside *et al.*, 1990; Peura *et al.*, 1991; Utsumi *et al.*, 1992) Typically, the sexing of embryos has been predicted by metaphase chromosome spread (Gimenez *et al.*, 1993), H-Y antibody (Wachtel *et al.*, 1988) or *in situ* hybridization (West *et al.*, 1987). However, all of these methods have drawbacks of be inaccurate or time-consuming. The polymerase chain reaction (PCR) is a breakthrough procedure for embryonal sex determination since it requires minimal amount of samples, no isotopes and a very short time. The PCR technology has been employed for sex determination of bovine preimplantation embryos (Peura *et al.*, 1991; Utsumi *et al.*, 1992; Kirkpatrick and Monson, 1993). Recently isolated and cloned Y chro-

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mosome-specific DNA has been used as a probe for embryo sex determination and it should be useful for sex determination of bovine pre-implantation embryos (Reed *et al.*, 1988, 1989; Miller and Koopmann, 1990; Kudo *et al.*, 1993). To establish an accurate and rapid sexing technique which permits the sexing of a large number of embryos within 4~5 hours, capillary polymerase chain reaction (PCR) was used to determine the sex of somatic cells, *in vitro* fertilized (IVF) or parthenogentic embryos and we used both Y-specific repeat sequences as targets for DNA amplification and bovine-specific repeat sequences as an internal control for the proper function of the PCR process.

MATERIALS AND METHODS

1. Preparations of embryos and genomic DNAs

Morulae or blastocyst stage embryos were obtained from *in vitro* fertilization (IVF) or 7% ethanol activation and subsequent coculture with bovine oviductal epithelial cells (BOEC). After removal of the zona pellucida-bound cumulus cells or residual sperms by 3% sodium citrate treatment, the embryos were treated with acid Tyrode's solution (pH 2.5) at 37℃ for 5 min to remove the zona pellucida. The resulting zona-free embryos were washed 3 times with Ca^{2+} and Mg^{2+}-free phosphate buffer solution (PBS) supplemented with 4 mg/mL bovine serum albumin (BSA), then transfered into 0.5mL Eppendorf tube containing 18 μL lysate buffer (10 mM Tris. HCl, pH 8.9, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, and 2% β-mercaptoethanol). After 3 times freezing (−196℃) and thawing (37℃), the genomic DNA of embryos was prepared by adding 0.2μg/μL proteinase K and incubating at 50℃ for 1h, followed by inactivation at 95℃ for 10 min. Genomic DNA extraction from fresh male bovine blood cells was carried out by the methods described in "Molecular Cloning" (Sambrook *et al.*, 1989). Isolated genomic DNAs for the use of template DNA of PCR were dialyzed in 4 liters of TE(pH 8.0) buffer using a dialysis membrane (Dialysis tubing, Sigma) for 24 hrs at 4 ℃.

2. Selection of primer sets for PCR

Two sets of bovine-specific and Y chromosome- specific primers were used in the sexing analysis. The sequence of the bovine-specific primers were 5'-TGGAAGCAAAG AACCCCGCT-3' and 5'-TCGTGAGAAACCGCACACTG-3' (Plucienniczak *et al.*, 1982). Bovine Y chromosome-specific primers were 5'-GGATCCGAGACAC AGAACAGG-3' and 5'-GCTAATCCATC CATCCTATAG-3'(Reed *et al.*, 1988). The length of bovine- and Y chromosome-specific amplification product was 216 and 301 bp, respectively.

3. PCR amplification

For PCR amplification, PCR buffer (10 mM Tris-HCl pH 8.9, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, and 0.01% BSA), each 200 μM of dNTPs (dATP, dGTP, dCTP and dTTP), 10 pmol bovine-specific primers, 40 pmol Y-specific primers and 2 U Taq DNA polymerase (Promega Co., Madison, USA) was added to the tubes containing each embryo in a final volume of 50μL reaction mixture. Before addition of the enzyme, the samples were overlaid with 20μL of mineral oil to prevent the evaporation of the reaction mixture. Then, the PCR amplification of samples was carried out in the thermal cycler (The MiniCycler, Model PTC-150, AB Technology Ltd., Pullman, USA). All samples were initially denatured at 95℃ for 5 min. This was followed by 40 cycles consisting of denaturation at 94℃ for 30 sec, annealing at 58℃ for 1 min, and extension at 72℃ for 1 min. After last cycle, the samples were kept at 72℃ for an additional

10 min to complete perfectly DNA synshesis.

4. Gel Electorphoresis and sex determination

The PCR products (10 μL) were characterized by electrophoresis on 3% NuSieve 3:1 agarose gels (FMC Bioproducts, Rockland, USA) in the bufer of TAE. After electrophoresis at 50 V for 50 min, the separated fragments were visualized directly by ethidium bromide (EtBr) staining under ultraviolet light illumination. The embryonic sample was sexed as female if only a 216 bp product was amplified. Sample with both 216 bp and 301 bp products was regarded as male.

RESULTS AND DISCUSSION

1. Sex determination in somatic cells

Since the number of embryonic cells to be used for PCR analysis is scarce (about 30~120 cells) it was necessary to determine an optimal condition to give a visible PCR product with a minimal amount of genomic DNA. Therefore, the amplification reactions first were performed using blood cell genomic DNAs serially diluted from 10 ng to 10 pg (except 0.5 ng) as intervals of 10 times. Fig. 1. shows the electrophoretic pattern of bovine-(216 bp) and Y chromosome-specific (301 bp) DNA fragments in PCR reactions containing various amounts of template genomic DNA, respectively. The bovine-specific fragments are clearly visible and give a constant and strong amplifying pattern regardless of the amounts of template DNA whereas the Y chromosome-specific fragments were hardly visible as the amounts of template DNA became less (diminished) gradually. It is considered to be due to the difference of repetitivity between bovine-specific repeated sequences and Y chromosome-specific repetitive sequences. Based on the fact that bovine-specific sequences were much more repetitive than Y chromosome-specific sequences as indicated in Fig. 1, the ratio of bovine-specific primers to Y-specific primers were adjusted to 1:4. Under the modified reaction conditions, the PCR amplification was carried out using 10 ng of blood cell genomic DNA. The positive and negative controls as well as a blank (10 ng male bovine DNA, zona pellucida, and no DNA, respectively) were also subjected to PCR amplification. As shown in Fig. 2, both bovine- and Y-specific products were clearly shown after 40 cycles. No fragment was detected in the no DNA and zona pellucida. Therefore, the results indicated that the contamination from non-embryonic sources of DNA was efficiently excluded by our routinely used wash procedure.

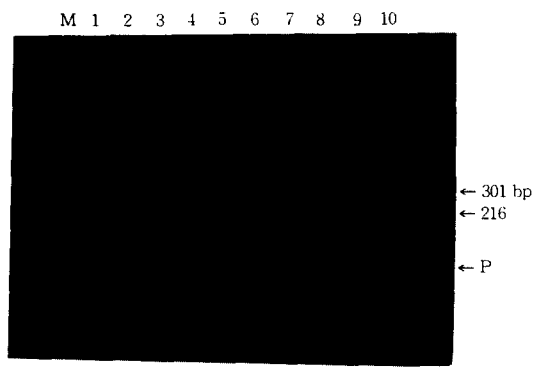

Fig. 1. Amplification of genomic DNA from somatic cells using bovine-specific primers and Y chromosome-specific primers, respectively. All the samples were amplified using genomic DNA from male blood cells at 10 ng, 1 ng, 0.5 ng, 0.1 ng and 10 pg, respectively. Lane M, molecular size marker (pBluescript SK digested with Hpa II); lane 1 through 5, male genomic DNA amplified in the presence of bovine-specific primers only; lane 6 through 10, male genomic DNA amplified in the presence of Y chromosome-specific primers only; p, primers.

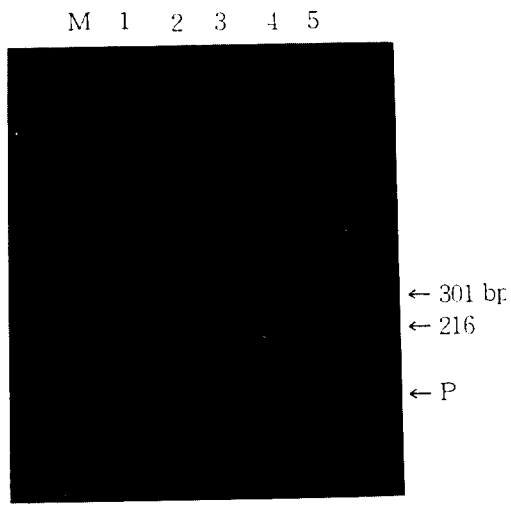

Fig. 2. Amplification of bovine- (216 bp) and Y chromosome-specific (301 bp) DNA in somatic cells. Lane M, molecular size marker (pBluescript SK digested with Hpa II); lane 1, negative control without DNA; lane 2, negative control with the zona pellucida (ZP) to know whether the contamination derived from granulosa cell and ZP-bound sperms occurs or not. : lane 3, gnomic DNA amplified with bovine-specific primers only; lane 4, genomic DNA amplified with Y chromosome-specific primers only; lane 5, genomic DNA amplified with both bovine- and Y chromosome-specific primers.

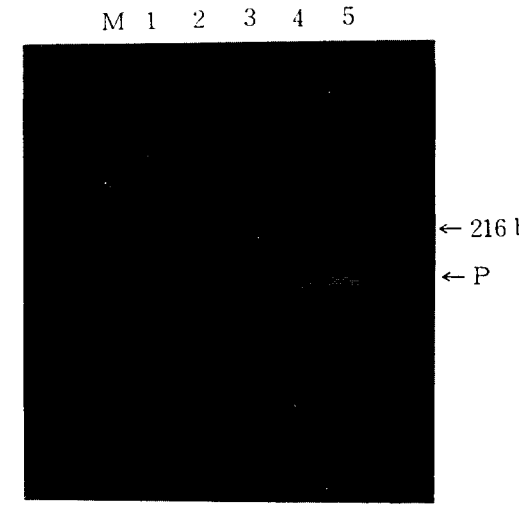

Fig. 3. Amplification of DNA from parthenogentic embryos. Lanes 1 through 3, embryo samples amplified with bovine-specific primers only; lane 4, embryo sample amplified with Y chromosome-specific primers only; lane 5, embryo sample amplified with both bovine- and Y chromosome-specific primers.

2. Embryonic sex determination

Unlike somatic cells, *in-vitro* produced embryos from follicular oocytes contain remnants of granulosa or cumulus cells and zona pellucida-bound spermatozoa which cause multiple amplified products. Therefore, granulosa cells and tightly bound spermatozoa should be completely removed for the accurate determination of sex in embryonic cells. The procedure employed in this study was 3% sodium citrate treatment followed by acid Tyrode's solution (pH 2.5) and the resulting zona-free embryos were subjected to PCR amplification. Because the inclusion of the control gene was of great importance in the accurate sex determination of embryos, we used Y chromosome-specific primers amplified in male only as targets for DNA amplification and bovine-specific primers amplified in both male and female as an internal control. Furthermore, the use of parthenogenetic embryos (XO or XX) was another new system to ensure accurate sexing because their sexes are certainly female without any sperm penetration. As shown in Fig. 3, no Y-specific product was found in the parthenogentic embryos. They always showed bovine-specific products (216 bp), thus serving as an excellent control for the reaction. However, normal embryos produced by IVF demonstrated bovine-specific product alone (216 bp; female) and Y-specific product alone (301 bp; male), and two products (216 and 301 bp; male) as in Fig. 4. Embryo samples were amplified in

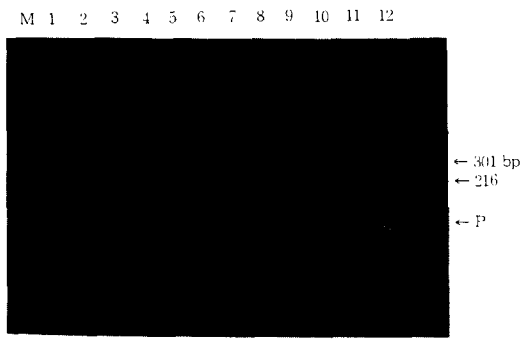

Fig. 4. Amplification of DNA from IVF embryos by capillary PCR. Lanes 1 through 4, embryo samples amplified with bovine-specific primers only; lanes 4 through 8, embryo samples amplified with Y chromosome -specific primers only; lanes 9 through 12, embryo samples amplified with both bovine- and Y chromosome-specific primers. Sample 5, 7 and 11 were classified as male embryo and sample 6 and 8 (no Y-specific product), 9, 10 and 12 were classified as female embryo, respectively.

the presence of bovine-specific primer only (lanes 1~5), Y chromosome-specific primers (lanes 6~8) and two sets of primers (lanes 9~12), respectively. These results show that the bovine Y chromosome-specific primers could be used as PCR sexing of preimplantation bovine embryos produced *in vitro*.

3. Embryonic sex ratio

Samples from blastocyst stage 25 embryos were successfully sexed by capillary PCR and the results are summarized in Table 1. The final procedure was used to examine the sex ratio of the IVF embryos. From a typical analysis, 10 (52.6 %) of total 19 embryos were classified as male and 9 embryos (47.4 %) were judged as female, indicating that the sex ratio of sexed embryos did not differ significantly from the expected 1:1 ratio. Six parthenogenetic embryos all gave bovine-specific product (female) alone.

Table 1. Representative sex predetermination of bovine preimplantation embryos by capillary PCR.

Sources of embryos	No. of embryos	No. of sexed embryos as Male (%)	Female (%)
Fertilzed	19	10 (52.6)	9 (47.4)
Parthenogentic	6	0 (0.0)	6 (100.0)

* Total 90 embryos were analysed, 65 embryos were used in the preliminary experiments to set an optimal PCR condition.

From these results, the established wash procedure, citrate treatment, removal of the zona pellucida and use of parthenogentic embryos should be practical in the predetermination of sex in early cattle embryos, preventing from contamination of other cell sources and ensuring the correct reaction at any single analysis.

SUMMARY

Predetermination of sex in early embryos is of great value in the industry of animal production since it provides a means to select animal sex of interest. Capillary polymerase chain reaction (PCR) was used to determine the sex of somatic cells, *in vitro* fertilized (IVF) or parthenogentic embryos. Morulae or blastocysts to be sexed were obtained from IVF and subsequent coculture with bovine oviductal epithelial cells. The embryonic DNA samples were prepared by suspending single embryos in PCR lysate buffer containing 0.2 $\mu g/\mu L$ proteinase K and incubated at 50℃ for 1h followed by inactivation at 95℃ for 10 min. Two amplified products (Y- and bovine-specific) were obtained in male samples whereas only one product (bovine-specific) in female and parthenogenetic embryos. The sex ratio (♂ : ♀ = 10 : 9) did not differ significantly from the expected 1:1 ratio by this technique. False sex determination was often found,

which may be associated with the residual cumulus cells or zona pellucida-bound sperms. Citrate treatment followed by complete removal of the zona pellucida eliminated false positive or negative results.

These results suggest that rapid (2h amplification) and accurate sexing is now possible in bovine embryos produced *in vitro*, and that the contamination from non-embryonic sources could be efficiently excluded by the established method.

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