Sex Determination by Polymerase Chain Reaction and Karyotyping of Bovine Embryos at First Cleavage *in vitro*

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ABSTRACT The purpose of this study was to compare the embryo sexing methods between PCR and karyotyping of *in vitro* produced (IVP) bovine embryos at the 2-cell stage. The embryos were divided into 3 groups according to the time of first cleavage completion. Zygotes cleaving before 24 hours postinsemination (hpi), during 24-36 hpi and after 36 hpi were classified as fast, intermediate and slow cleaving embryos, respectively. All 2-cell stage bovine embryos were separated into two blastomeres, one was karyotyped and the other was subjected to PCR amplification. The percentages of embryos which were sexed by PCR were significantly ($p<0.005$) higher than those analyzed by karyotyping (100% vs 56%). The proportions of male embryos sexed by PCR and karyotyping in the fast and intermediate cleaving groups were significantly ($p<0.05$) higher than female embryos, whereas the proportions of males were significantly ($p<0.05$) lower in the slow cleaving group. The overall ratios of males to females analyzed by both methods were significantly ($p<0.05$) different from the expected ratio of 1:1.

KEYWORDS: embryo sexing, PCR, karyotyping, first cleavage, *in vitro* production.

INTRODUCTION

Sex determination of preimplantation embryos is important for controlling sex ratios of domestic animals as well as in human for the diagnosis of X-linked genetic disorder at an early stage of embryonic development, enabling the selection and transfer of healthy conceptus.[1,2] Previous studies have suggested that a higher occurrence of males was demonstrated among more advanced embryonic stages whereas the preference for females was found among less advanced stages. Pavasuthipaisit *et al*[3] determined the sex of Day 7 *in vitro* produced bovine embryos (IVP) by immunofluorescence and showed that fast developing embryos were predominantly male while the slower ones were female. Avery *et al*.[4] reported that *in vivo* produced bovine male embryos developed faster into morulae and blastocysts than did female embryos as sexed by karyotyping. Polymerase chain reaction (PCR) sexing of bovine IVP embryos at Day 7 also showed that fast developing embryos from both full expanding and hatched blastocysts were predominantly males.[5] However, sex detection of embryos as early as the 2-cell stage would be more informative and beneficial in embryo sex selection. Recently, Saikhun *et al*[6] found that the first cleavage occurred faster in male than female bovine IVP embryos analyzed in the 2-cell stage by cytological sexing. Although the accuracy of the karyotyping method is high, its weakness is the low percentage of embryos that can be sexed because the analyzable metaphase plates that can be prepared are in relatively low numbers. In contrast, the PCR method has been shown to yield high percentage of embryos being sexed.[7,8,9] The objective of the present study was therefore to determine the sex of IVP bovine embryos in the 2-cell stage according to the time of first cleavage completion by using PCR as compared to karyotyping.

MATERIALS AND METHODS

Embryo production

The bovine embryos were produced *in vitro* by methods previously described.[10] Briefly, the ovaries were collected at an abattoir from cows and heifers within 30 min of slaughter, maintained in NaCl at 30-35°C and transported to the laboratory within 1 to 3 h after collection. Cumulus-oocyte complexes (COCs) were collected by aspiration from 2-6 mm antral follicles and matured in TCM 199 containing LH, FSH, estradiol, gentamycin, pyruvate and 10%

FCS at 39°C for 24 h. COCs were fertilized with $1x10^6$ cells/ml of frozen-thawed spermatozoa in 50 µl/droplet of IVF-TALP medium under the condition of 5% CO_2 in air at 39°C. After 20 h co-incubation of spermatozoa and oocytes, presumptive zygotes were co-cultured with bovine oviductal epithelial cells. Two-cell embryos were collected at 24, 30, 36, 42 and 48 hours postinsemination (hpi) from cultured drops. Zygotes cleaving before 24 hpi, during 24-36 hpi and after 36 hpi were classified as fast, intermediate and slow cleaving embryos, respectively. All cleaved (2-cell) embryos presented at each point of time were treated with 0.1% pronase in phosphate buffer saline (PBS) for 1 min to digest zona pellucida with attached sperm. Pair blastomeres of the zona-free two-cell embryos were separated by gentle pipetting using a glass micropipette. One blastomere was subjected to karyotyping and the other for PCR amplification.

Embryo sexing by karyotyping

The blastomeres were processed and chromosomes were prepared by the method described in the previous study.[11] The blastomeres were incubated in a TCM 199 medium containing 0.08 µg/ml of colchicine for 8-10 h to stop mitosis at metaphase and then treated with hypotonic solution of 1% sodium citrate for 10 min and placed on a clean grease-free glass slide. The slide was treated with a fixative mixture of methanol and acetic acid with a ratio of 1:1, stained with 5% Giemsa (Sigma, St Louis, MO,USA) at pH 6.8 for 10 min. The metaphase spread was located and the chromosomes were counted under the microscope at x200. The sex chromosomes were examined at x1000 and the sex of the embryo was diagnosed as described in the previous study.[12]

Embryo sexing by PCR

The blastomeres were washed in PBS and then transferred into a 0.6 ml microcentrifuge tube containing 40 ml of 5% (w/v) Chelex 100 (Biorad, Richmond, CA, USA). The cells were lysed by boiling at 100°C for 10 min, mixed by vortex for 15 sec, and stored at -20°C until assayed.[13] The samples were thawed at room temperature and centrifuged at 12,000 rpm for 3 min prior to mixing with PCR reagents. Two sets of PCR primers were used to determine their sex; Y-chromosome specific primers (BRY 1a)[14] and bovine specific satellite sequence primers.[15] The amplification reactions were conducted in a total volume of 50 µl containing PCR buffer, 200 mM of dATP, dCTP, dGTP and dTTP, 0.5 unit of Taq DNA polymerase and 2 pairs of primers (25 µM BRY 1a, 5 µM satellite sequence). The amplification was carried out in a DNA Thermal cycler (Perkin Elmer Cetus) for 40 cycles at 94°C for 30 sec, 58°C for 45 sec and 72°C for 45 sec followed by 1 cycle of 72°C for 10 min. Ten microliters of each PCR product were analyzed on 2% agarose gel and ethidium bromide staining. The gel was visualized under ultraviolet illumination for the positive 300 bp band of BRY 1a and 216 bp of the satellite sequence. The samples which revealed both bands were assigned as male while the samples giving only a satellite sequence band were assigned as female.

Statistical analysis

A chi-square analysis using 2x2 contingency table was performed to compare sex ratios between methods, while a chi-square goodness of fit was used to compare sex ratios with an expected ratio of 1:1.

Results

In the present experiment, a total of 448 oocytes were inseminated, of which 302 (67%) cleaved to the 2-cell stage (Fig 1A). Most of the fertilized oocytes cleaved during 24 to 36 hpi (179/302, 59%) while 42/302 (14%) cleaved before 24 hpi and 81/302 (27%) cleaved after 36 hpi (Table 1). All cleaved oocytes (2-cell) were treated by pronase to remove zona pellucida; the blastomeres were separated for karyotyping and PCR sexing (Figs 1B and 1C).

Of the 302 fixed blastomeres, the sex of 170 (56%) embryos could be determined by karyotyping (Table 1). Ninety-eight (58%) embryos were male and 72 (42%) were female. The karyotypes of female and male embryos are shown in Figs 2 and 3, respectively. The overall sex ratio of male : female (1.4:1.0) were significantly ($P<0.05$) higher than the expected 1:1. The sex ratios of fast, intermediate and slow cleaved embryos using karyotyping were 2.9:1.0, 1.9:1.0 and 0.5:1.0, respectively. Chromosomal anomalies were found in 46 (15%) of the fixed embryos. The number of fixed embryos that could not be sexed due to either the absent cells in metaphase (56/302, 19%) or poor metaphase appearance (30/302, 10%) were relatively high.

Fig 4 shows the agarose gel electrophoresis from the PCR amplification of the single blastomere derived DNA. Lanes showing both a 150 bp male-specific fragment and a 216 bp bovine DNA-specific fragment were considered as male embryos. If only the 216 bp DNA-specific product was visible, the

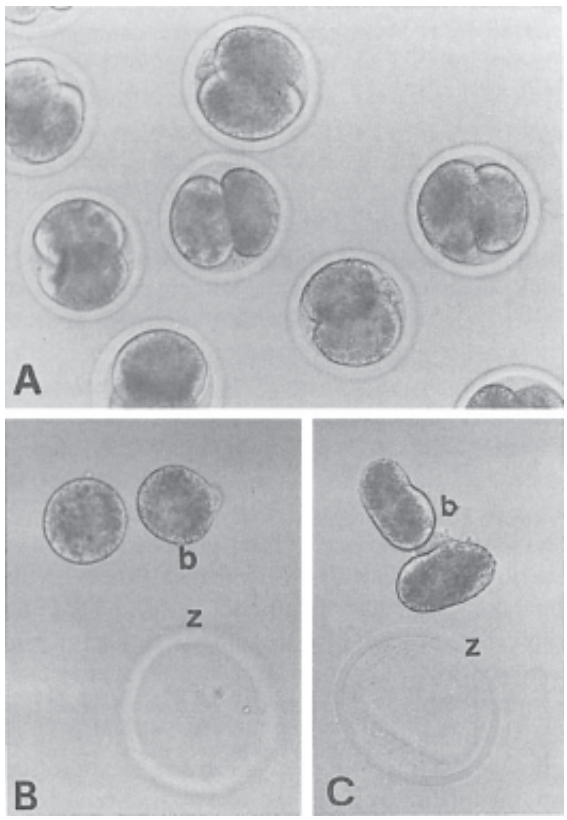

Fig 1. The 2-cell stage bovine embryos (A) and blastomeres after zona pellucida removal (B,C), z: zona pellucida, b: blastomere.

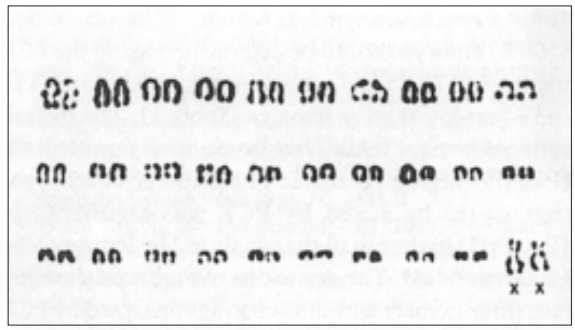

Fig 2. Karyotype of female bovine embryo with 60 XX.

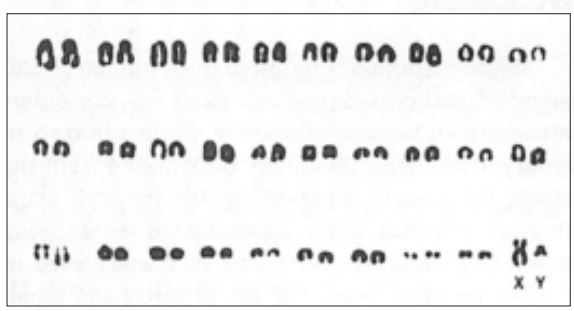

Fig 3. Karyotype of male bovine embryo with 60 XY.

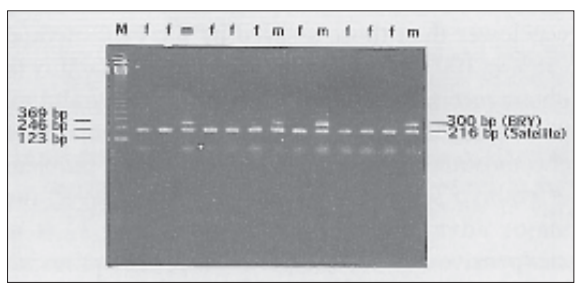

Fig 4. Sex determination of single blastomere separated from the 2-cell bovine embryo visualized under UV transilluminator Lane 1, 123 bp ladder DNA; Lanes 4,8,10 and 14, male embryo samples in which both 300 and 216 bp bands were amplified. Lanes 2,3,5,6,7,11,12 and 13, female embryo samples in which only 216 bp bands were amplified.

Table 1. Outcome of the 2-cell bovine embryo sexing categorized according to the time of first cleavage completion.

Time of 2-cell stage selection (hpi)	No of embryos	No.of embryos PCR			No.of embryos Karyotyping		
		Sexed	M	F	Sexed	M	F
24	42	42	30	12	27	20	7
30	101	101	67	34	52	33	19
36	78	78	52	26	45	30	15
42	45	45	13	32	24	8	16
48	36	36	9	27	22	7	15
Total	302	302	171	131	170	98	72

M: male, F: female

embryos were assigned as females. The sex of 302 (100%) embryos could be determined using the PCR method : 171 (57%) embryos were males and 131 (43%) embryos were females (Table 1). The overall ratio of male : female embryos was significantly ($P<0.05$) higher than 1:1. The number of embryos that could be sexed by PCR was significantly ($P<0.005$) higher than that analyzed by karyotyping (100% vs 56%). The sex ratios of embryos showing fast, intermediate and slow cleavage analyzed by PCR were 2.5:1.0, 2.0:1.0 and 0.4:1.0, respectively.

Discussion

Single blastomeres biopsied from human[1,16] and mice[17,18] embryos have been used for sex determination. The sex of mouse preimplantation embryos has been accurately determined from the single blastomere separated at the two-cell stage through detection of the Y-specific sequences using a two-step PCR method.[17] The PCR assay used in the present study was accurate, sensitive and rapid. It took at least 8 h for karyotyping whereas the PCR sexing could be carried out in 3 h. The number of embryos sexed by karyotyping in the present study was lower than those assayed by the PCR method (56% vs 100%). This could be due to the inability to obtain metaphase from all preparations. In addition, poor spreading and over scattering of metaphase chromosomes have been noted as a major problem of embryo sexing by karyotyping.[19] However, the major advantages of karyotyping are; 1) it is inexpensive and easy to perform, 2) it requires no sophisticated equipment, and 3) it can identify chromosomal anomalies before the embryos are transferred. The percentages of bovine embryos at 5 days after insemination which could be sexed by karytyping are similar in the cell numbers ranging from 4 to 95.[20] Embryo sexing by both PCR and karyotyping in the present study demonstrated that male bovine IVP embryos had completed the first cleavage faster than female embryos. Our finding should be beneficial in embryo sexing if 2-cell embryos are selected according to their cleavage rate for further culture *in vitro*. Yadav *et al.*[20] separated the 2-cell IVP bovine embryos according to the time when they completed their first cleavage and karyotyped at 5 days after insemination. They showed that the first cleavage occurred faster in the male than in the female embryos. Dominko and First[21] also observed a predominance of males among fast cleaved bovine embryos.

Differential growth rate of male and female IVP bovine embryos at the 2-cell stage may be explained by several possibilities. The faster growth in male embryos may well be a consequence of the faster gene expression caused by Y-chromosomal genes. A possible effect of Y-linked genes promoting the rapid growth of male embryos has been suggested to be caused by H-Y antigen[4] or Y-chromosome growth factors.[22,23,24] It has been suggested that some Y-linked genes are actively transcribed before embryonic genome transcriptions occurs in bovine embryos during the 2-cell stage[25] which affect more rapid cleavage of the male embryos. Pergament *et al.*[26] have reported that Y-chromosome factor influences the rate of embryonic growth immediately after fertilization. Also, the faster growth of male bovine embryos may be due to Y-sperm activate embryonic genome to transcribe growth factors before embryonic transcription which increases the rate of cell division in XY embryos.[20] Sex related metabolic difference may be involved as a cause or effect because both bovine and human male embryos showed an increased metabolic rate compared with the female embryos.[27,28] Total glucose metabolism was found significantly greater in male than in female bovine blastocysts which might be related to more rapid development of male embryos.[27] Moreover, *in vitro* condition might account for the faster growth of male embryos because they cleaved faster than female embryos when cultured in the presence of glucose.[29] These lines of evidence support the findings of differences in sex ratio of the 2-cell bovine embryos reported in the present study. However, it should also be mentioned that Beyhan *et al*[30] found that the penetration rate of sorted Y-sperm was higher than X-sperm (44.7% vs 39.5%). Thus, the differences could have been existed before cleavage.

In summary, our results indicated that; 1) the success of sexing of single blastomere separated from the 2-cell stage IVP bovine embryos by PCR was higher than those analyzed by karyotyping, and 2) male bovine embryos cleaved to the 2-cell stage faster than female. The higher occurrence of males among fast cleaving embryos and of females among slow cleaved embryos could be used as an easy noninvasive method for embryo sexing. However, the viability of the embryos develops to transferable stages after selection and their sex determination

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REFERENCES

1. Handyside AH, Pattinson JK, Penketh RJA, Delhanty JDA, Winston RML, and Tuddenham EGD (1989) Biopsy of human preimplantation embryos and sexing by DNA amplification. *Lancet* **1**, 347-9.
2. Penketh RJA, Delhanty JDA, Van Den Berghe JA, Finklestone EM, Handyside AH and Malcolm S (1989) Rapid sexing of human embryos by non-radioactive in situ hybridization : potential for preimplantation diagnosis of X-linked disorders. *Prenatal Diagn* **9**, 489-500.
3. Pavasuthipaisit K, Kitiyanant Y and Tocharus C (1993) Sex determination of in vitro produced bovine embryos by embryo developmental stage and immunofluorescence. *Thai J Physiol Sci* **6**, 63-74.
4. Avery B and Schmidt M (1989) Sex determination of bovine embryos using H-Y antibodies. *Acta Vet Scand* **30**, 155-64.
5. Carvalho RV, Del Campo MR, Plante T and Mapletoft RJ (1995) Effects of storage or development on sex ratio and survival after freezing of Day 7 bovine IVF embryos. *Theriogenology* **43**, 183-9
6. Saikhun J, Kitityanant Y, Chuangsoonneon U, Jaruansuwan M and Pavasuthipaisit K (1997) Difference of first cleavage rates and sex ratio of bovine embryos produced in vitro using chromosome analysis. *Thai J Physiol Sci* **10**, 37-49.
7. Schroder A, Miller JR, Thomsen PD, Roschlau K, Avery B, Poulsen PH, Schmidt M and Schwerin M (1990) Sex determination of bovine embryos using the polymerase chain reaction. *Anim Biotechnol* **1**, 121-33.
8. Peura T, Hyttinen JM, Turunen M and Janne J (1991) A reliable sex determination assay for bovine preimplantation embryos using the polymerase chain reaction. *Theriogenology* **35**, 547-55.
9. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB and Ehrlish HA (1989) Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487-91.
10. Kitiyanant Y, Thonabulsombat C, Tocharus C, Snitwongse B and Pavasuthipaisit K (1989) Co-culture of bovine embryos from oocytes matured and fertilized in vitro to the blastocyst stage with oviductal epithelial tissue. *J Sci Soc Thai* **15**, 251-60.
11. King WA (1984) Sexing embryo by cytological method. *Theriogenology* **21**, 7-17.
12. King WA, Yadav BR, Xu KP, Picard PL, Sirard MA, Verini SA and Betteridge KJ (1991) The sex ratios of bovine embryos produced *in vivo* and *in vitro*.*Theriogenology* **36**, 779-88.
13. Gutierrez-Adan H, Cushwa WT, Anderson GB and Medrano JF Ovine–specific Y-chromosome RAPD-SCAR marker for embryo sexing. *Animal Genetics* **28**, 135-8.
14. Reed KC, Matthaei KI, Mann DA, Beaton S and Matthews MA (1989) Determination of genetic sex in ruminants using Y-chromosome specific polynucleotides. Patent Coorperation Treaty No.WO 89/07154.
15. Plucienniczak A, Skowronski J and Jaworski J (1982) Nucleotide sequence of bovine 1.715 satellite DNA and its relation to other bovine satellite sequences. *J Mol Biol* **158**, 293-304.
16. Liu J, Lissens W, Devracy P, Steirteghem AV and Liebaers I (1994) Amplification of X- and Y-chromosome specific region from single human blastomeres by polymerase chain reaction for sexing of preimplantation embryo. *Hum Reprod* **9**, 716-20.
17. Kunieda T, Xian M, Kobayashi E, Imamichi T, Moriwaki K and Toyoda Y (1993) Sexing of mouse preimplantation embryos by detection of Y chromosome-specific sequences using polymerase chain reaction. *Biol Reprod* **46**, 692-7.
18. Gimenes C, Egozeur J and Vidal F (1994) Sexing sibling mouse blastomeres by polymerase chain reaction and fluorescent in situ hybridization. *Hum Reprod* **9**, 2145-9.
19. Picard L, King WA and Betteridge KJ (1985) Production of sexed calves from frozen-thawed embryos. *Vet Rec* **117**, 603-8.
20. Yadav BR, King WA and Betteridge KJ (1993) Relationships between the completion of first cleavage and the chromosomal complement, sex, and developmental rates of bovine embryos generated *in vitro*. *Mol Reprod Dev* **36**, 434-9.
21. Dominko T and First NL (1993) Male predominance of bovine embryos can be observed at the 2-cell stage. *Biol Reprod* **48**, 439 (abstr).
22. Burgoyne PS (1993) A Y-chromosomal effect on blastocyst cell number in mice. *Development* **117**, 341-5.
23. Zwingman T, Erickson RP, Boyer T and Ao A (1993) Transcription of the sex-determining region genes Sry and Zfy in the mouse preimplantation embryo. *Proc Natl Acad Sci* **90**, 814-7.
24. Xu KP, Yadav BR, King WA and Betteridge KJ (1992) Sex-related differences in developmental rates of bovine embryos produced and cultured in vitro. Mol Reprod Dev 31, 249-52.
25. Memili E, Dominko T and First NL (1998) Onset of transcription in bovine oocyte and preimplantation embryos. *Mol Reprod Dev* **51**, 36-41
26. Pergament E, Fiddler M, Cho N, Johnson D and Holmgren WJ (1994) Sexual differentiation and preimplantation cell growth. *Hum Reprod* **9**, 1730-2.
27. Tiffin GJ, Rieger D, Betteridge KJ, Yadav BR and King WA (1991) Glucose and glutamine metabolism in pre-attachment cattle embryos in relation to sex and stage of development. *J Reprod Fertil* **93**, 125-32.
28. Ray PF, Conaghan J, Winston RML and Handyside AH (1995) Increased number of cells and metabolic activity in male human preimplantation embryos following in vitro fertilization. *J Reprod Fertil* **104**, 165-71.
29. Bredbacka KS and Bredbacka P (1996) Glucose controls sex-related growth rate differences of bovine embryos produced *in vitro*. *J Reprod Fertil* **106**, 169-72.
30. Beyhan Z, Johnson LA and First NL (1999) Sexual dimorphism in IVM-IVF bovine embryos produced from X and Y chromosome bearing spermatozoa sorted by high speed flow cytometer. *Theriogenology* **52**, 35-48.