Acta vet. scand. 1991, *32*, 283–286.

Brief Communication

Birth of Calves Developed from Embryos of Predetermined Sex

New methods for predetermination of the sex of preimplantation embryos offer considerable economical value when applied to embryo transfer industry. Some of these methods can already be used on-farm conditions (*Herr et al.* 1990). We recently described a polymerase chain reaction (PCR) based sexing assay for bovine embryos (*Peura et al.* 1991a). By using this method we achieved 100 % accuracy in embryonal sex determination as judged by the birth of 8 consequtive calves of predetermined sex.

Bovine embryos were produced completely in vitro by the method described by *Peura et al.* (1991b). Briefly, cow ovaries were obtained from a local slaughterhouse (Lihapolar, Kuopio), and immature bovine oocytes were aspirated from antral follicles. Maturation was achieved in TCM199-medium supplemented with 5 % fetal calf serum, 5 % estrous cow serum, 2 mmol/l L-glutamine, 0.25 mmol/l sodium pyruvate, 20 μg/ml gentamycin, 2 μg/ml FSH, 10 μg/ml LH and 1 μg/ml estradiol-17β. After 22–24 h of maturation oocytes were fertilized with frozen-thawed bull sperm. One day after fertilization oocytes were transferred with intact cumulus cells to 50 μl drops of frozen-thawed bovine oviducal epithelial cell-conditioned medium under paraffin oil for further culture. All cultures were performed in humified incubator at +39°C and 5 % CO_2 in air.

Five, 6 or 7 days after fertilization some morulas and blastocysts were bisected using Eppendorf Micromanipulator 5170. One of the resulting demi-embryos was always cultured further in CM-drop for 20 h, whereas the other demi-embryo was immediately put into a 50 μl lysate buffer (15 mmol/l Tris-HCl pH 8.9, 50 mmol/l KCl, 2.5 mmol/l $MgCl_2$, 0.1 % Triton X-100, 150 μg/ml proteinase K) and digested for 30–60 min at +37°C in order to make DNA amplifiable. The digestion was halted by denaturating proteinase K for 8 min at +99°C.

Sex determination of bovine embryo samples was performed according to our recently published procedure (*Peura et al.* 1991a) with slight modifications. Briefly, PCR reaction was carried out in the PCR-buffer (lysate buffer without proteinase-K) containing 0.2 mmol/l dNTP (Pharmacia LKB Biotechnology, Uppsala, Sweden), 1.25 U Taq DNA polymerase (Promega, Madison, WI, USA) and 5–20 pmol of primers in total volume of 50 μl.

One pair of bovine-specific and two different pairs of Y chromosome-specific primers were used. The 216-basepair long PCR-product from bovine satellite 1.715 (*Pluciennicak et al.* 1982) indicated the presence of the sample. The 301-basepair long PCR-products from bovine Y-chromosome repeat BRY.1 (*Reed et al.* 1988) and BRY.4a (*Reed at al.* 1989) indicated the sex of the embryo. Because the bovine satellite 1.715 is much more repetitive than either BRY.1 or BRY.4a both Y chromosome-specific primers were used to make the system more sensitive and reliable in Experiment 2.

In Experiment 1 the sexing was carried out in one PCR-reaction with bovine satellite

and BRY.1 specific primers. In Experiment 2 the embryo lysates were divided into 2 aliquots and 2 separate PCR-reactions were carried out. The first aliquot was analysed with bovine satellite and BRY.4a specific primers and the other aliquot with BRY.1 and BRY.4a specific primers. Positive controls (bull and cow blood lysate samples) and negative controls (biopsy material replaced by PBS) were included in every assay performed.

The amplifications were carried out in a Hybaid thermal reactor (Hybaid Ltd., Teddington, Middlesex, UK) by first denaturating samples at + 96°C for 3 min followed by 48 (Experiment 1) or 34 (Experiment 2) cycles each consisting of denaturation at + 95°C for 1 min, annealing at + 58°C for 1 min and primer extension at + 72°C for 30–60 s. After the last cycle samples were incubated further at + 72°C for 5 min. Amplification products were then electrophoresed on a 2.5 % agarose gel, stained with ethidium bromide and visualized under ultraviolet light.

After an overnight culture the viability of the demi-embryos in culture was assessed and embryos for transfer were selected according to their morphological appearance and sex. Transfers were performed non-surgically to synchronized recipients at the Veterinary Research Station of University of Kuopio.

Total of 79 embryos were bisected in 2 different experiments. Despite the differencies in the actual sexing assays between experiments the results were very similar in both cases. The total survival rate after overnight culture was 53 % (55 % and 52 % in Experiment 1 and 2, respectively), and the percentage of successful sexing assays was 80 % (79 % and 80 %, respectively). In most cases failure was attributable to sample loss. From the analysed embryos 44 % (48 % and 43 %, respectively) were female and 56 % (52 % and 57 %, respectively) male. For the transfers 6 female and 4 male embryos were chosen and transferred to the 5 recipients in Experiment 1, and 6 female and 2 male embryos to the 4 recipients in Experiment 2, each recipient receiving 2 embryos of the same sex. Seven recipients became pregnant (4 and 3 in Experiment 1 and 2, respectively) resulting total pregnancy rate of 78 %. Three heifer calves and 1 bull calf were born from both experiments (Table 1). One of the pregnancies in Experiment 2 was a twin pregnancy producing 2 heifer calves. One anatomically well formed and normal singleton heifer calf died shortly after birth. The cause of death could not be solved in necropsy at National Veterinary Institute. All of the calves were of predicted sex.

Availability of sex determined embryos made transfer of 2 demi-embryos of the same sex into each recipient possible, thus increasing the possibility of pregnancy. Without sex determination transfer of 2 embryos would not be recommended because of the high risk of freemartinism. In previous studies it has been found out that transfer of more than 1 (demi-)embryo at the time increases likelyhood of pregnancy possibly be-

Table 1. The pooled results of embryo bisections, sexing assays and embryo transfers in Experiments 1 and 2.

Bisected embryos	Embryos surviving bisection	Sexed embryos	Male	Female	Recipients	Demi-embryos transferred	Calves born	Calves with correct sex
79	45/79 (53 %)	63/79 (80 %)	35/63 (56 %)	28/63 (44 %)	9	18	8	8 (100 %)

cause of the stronger embryotrophic signal for the recognition of pregnancy. In the work of *Picard et al.* (1985) the transfer of 2 fresh demi-embryos (both halves of 1 bisected embryo) into each recipient resulted 13 calves from 20 demi-embryos (65 % calving rate). In the same experiment also single demi-embryos were transferred resulting in only 3 calves from 8 demi-embryos (38 % calving rate). In the work of *Herr et al.* (1991) 16 of 30 biopsied embryos (53.5 %) resulted in pregnancies (detected at 90 days). All embryos in both of these experiments were produced in vivo. Also *Kippax et al.* (1991) had transferred both halves of 1 bisected embryo into each recipient receiving 35 %, 63 % and 43 % foetuses/demi-embryo (demi-embryos either both in the original zona pellucida; or 1 in and 1 out of the zona pellucida; or both out of the zona pellucida, respectively). In our experiments no attempts were made to hold demi-embryos within zona pellucida, and so practically all of the demi-embryos were transferred without zona. Calving rate in this experiment with in vitro produced embryos was 44 % (8 calves/18 demi-embryos transferred). The overall pregnancy rate was 78 % (7 out of 9 recipients pregnant).

Only limited comparative data about the accuracy of different embryo sexing methods, as judged from the foetuses/calves, is available. However, good accuracy has been achieved with PCR-method in the work of *Herr et al.* (1990), where 11/12 calves (91.6 %) were of predicted sex. The accuracy of the method can also be studied by analysing several sections from 1 embryo. With our method we have achieved 100 % accuracy (11 embryos sectioned into 4 sections, 1 embryo sectioned into 2 sections) from the analysable samples (*Peura et al.* 1991a). Based on the accuracy of the results achieved with transfers of analysed embryos as well as with sectioned embryos, our method appears to be extremely reliable for sex determination of bovine preimplantation embryos. Possibility to transfer more than 1 embryo to a recipient increases likelyhood of pregnancy and decreases the number of recipients needed.

Acknowledgements

This work was financially supported by the Ministry of Agriculture and Forestry of Finland and the Al Station for Eastern and Middle Finland. The authors want also to express gratitude to NIDDK for providing porcine FSH and LH.

T. Peura, J.-M. Hyttinen, M. Turunen, J. Aalto, V. Rainio and *J. Jänne*
Departments of Anatomy, Biochemistry & Biotechnology and the Veterinary Research Station, University of Kuopio, Finland.

References

Herr CM, Holt NA, Matthaei KI, Reed KC: Sex of progeny from bovine embryos sexed with a rapid Y-chromosome-detection assay. Theriogenol. 1990, *33,* 247.

Herr CM, Matthaei KI, Bradley MP, Reed KC: Rapid, accurate sexing of livestock embryos. In: WG Hill, R. Thompson, JA Woolliams (eds.) "Proceedings of the 4th World Congress on Genetics Applied to Livestock Production", Edinburgh, United Kingdom, 23–27.7 1990. Vol. XIV, p. 334–343.

Herr CM, Reed KC: Micromanipulation of bovine embryos for sex determination. Theriogenol. 1991, *35,* 45–54.

Kippax I, Christie W, Rowan TG: Effects of method of splitting, stage of development and presence or absence of zona pellucida on foetal survival in commercial bovine embryo transfer of bisected embryos. Theriogenol. 1991, *35,* 25–35.

Peura T, Hyttinen J-M, Turunen M, Jänne JA: A reliable sex determination assay for bovine preimplantation embryos using the polymerase chain reaction. Theriogenol. 1991a, *35,* 547–555.

Peura T, Hyttinen J-M, Jänne JA, Aalto J: Transfer of in vitro matured, fertilized and cultured bovine embryos of predetermined sex. Theriogenol. 1991b, *35,* 254.

Picard L, King WA, Betteridge KJ: Production of sexed calves from frozen-thawed embryos. Vet. Rec. 1985, *117,* 603–608.

Pluciennicak A, Skowronski J, Jaworski J: Nucleotide sequence of bovine 1.715 satellite DNA and its relation to other bovine satellite sequences. J. Mol. Biol. 1982, *158,* 293–304.

Reed KC, Matthaei KI, Mann DA, Beaton S: Determination of genetic sex in ruminants using Y-chromosome-specific polynucleotides. Patent Cooperation Treaty No. WO 89/07154, 1989.

Reed KC, Matthews ME, Jones MA: Sex determination in ruminants using Y-chromosome specific polynucleotides. Patent Cooperation Treaty No. WO 88/01300, 1988.

(Received May 31, 1991; accepted September 19, 1991).

Reprints may be requested from: T. Peura, Department of Anatomy, University of Kuopio, P. O. Box 1627, SF-70211, Kuopio, Finland.