Sex determination in bisected bovine embryos and conception rate after the transfer of female demi-embryos

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ABSTRACT: The aim of this study was to evaluate the efficiency of sex determination after microsurgical splitting of D_7 (Day 7) bovine embryos and to test the conception rate after subsequent transfer of female demi-embryos. High-quality morulae (n = 100) and early blastocysts (n = 123) obtained from superovulated donors were microsurgically bisected and blastomeres biopsied from one half of bisected embryos were analysed by PCR using specific primers for the Y-chromosome determinant. The female demi-embryos were transferred (ET) in pairs (bilateral) or individually (ipsilateral) to synchronized recipients. Sex determination was successfully completed in 92% of morulae (42.4% female) and 89.4% of early blastocysts (43.6% female). Conception rates were 56.5% (30.4% identical twins) and 48.8% (19.5% identical twins) after bilateral (n = 46) and ipsilateral (n = 82) transfers, respectively. The number of foetuses in relation to the number of original embryos before splitting was 40/46 (87%) and 40/41 (97.6%) after bilateral and ipsilateral transfers of demi-embryos, respectively. The results document that the microsurgical bisection in combination with PCR sex analysis represents a rapid and reliable approach to increase an amount of sex-desired calves in embryo transfer programs.

Keywords: cattle; embryo; superovulation; splitting; sexing; polymerase chain reaction; embryo transfer

The embryo transfer technology represents a powerful tool for the acceleration of various breeding programs in cattle. Known sex of embryos produced for use in ET programs can more effectively help to manage producer resources because more heifer calves per ET can be produced. This approach can improve the genetic potential of cattle herds in shorter time intervals.

The first serious attempt to realize this intention was made by Edwards and Gardner (1967) with rabbit blastocysts according to the presence of Barr bodies. Many methods for sex determination were used in farm animals later but they were time consuming and with high variability of results. Employed methods include cytogenetic analysis (Singh and Hare, 1980; Rall and Leibo, 1987), immunological assay (White et al., 1982; Booman et al., 1989), detection of metabolic differences between male and female embryos (Williams, 1986; Monk and Handyside, 1988), analysis of chromatin with Y-specific DNA probes (Leonhard et al., 1987; Bondioli et al., 1989; Kobayashi et al., 1998) and analysis on the basis of differences in cleavage rates (Avery et al., 1989). Better results were published when the PCR amplification of specific DNA sequences was used to determine the embryonic sex in cattle (Herr et al., 1990; Schroder et al., 1990; Peura et al., 1991), pigs (Pomp et al., 1995),

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horses (Peippo et al., 1995), humans (Handyside et al., 1990) and mice (Han et al., 1993). The recently published results support the efficiency of PCR method for sex determination in cattle with a high accuracy and in an acceptable time intervals (Thibier and Nibart, 1995; Lopes et al., 2001; Ekici et al., 2006; Yu et al., 2006). Flushed embryos from superovulated donors are almost exclusively used for the determination of sex in farming conditions. Our recent results show (Lopatarova et al., 2006) that nearly all flushings contain embryos of very different quality. For sexing the embryos of high morphological quality are desirable but they are often in the minority.

Sperm sexing and separation into X- and Y-bearing fractions for purposes of insemination or *in vitro* production of embryos (IVP) also seems to be a promising procedure in sex determination. However, actual publications show that the procedure for the capacitation of flow cytometry sorted sperm might be successfully applied for fresh sperm in IVP programs (Katska-Ksiazkiewicz et al., 2005) but the sorted frozen/thawed fractions of sperm poorly survived after the capacitation treatment (Katska-Ksiazkiewicz et al., 2006). Moreover, only few spermatozoa could be recovered following Percoll's separation. In the case of strict bull selection, the developmental capacity of IVP embryos is still lower in comparison with their *in vivo* counterparts (Galli et al., 2003) and may be numerically further reduced when sorted sperm is used in fertilization (Zhang et al., 2005). In field experiments when X chromosome-bearing fractions of sorted sperm were used for insemination, 86.5% accuracy (45 females out of 52 calves born) was observed (Smorag et al., 2005). The pregnancy rates with low doses of sexed sperm drop about 10–40% compared to conventional doses of non-sexed sperm (Schenk et al., 2006), and it may be due to procedures associated with sorting. In superovulation programs, the numbers of recovered transferable embryos were significantly higher when donors were inseminated with the non-sexed sperm than with the sexed inseminates (Sartori et al., 2004; Schenk et al., 2006).

The aim of this study is to estimate the validity of a protocol comprising bisection of high-quality embryos, biopsy for embryonic cells for sex determinations and subsequent transfer of demi-embryos in a bilateral or ipsilateral mode. The implementation of high-quality demi-embryos of desired sex in commercial ET programs is discussed.

MATERIAL AND METHODS

Animals and treatment

Selected donors (n = 60) were stimulated according to the protocol described by Holy et al. (1990). Briefly, Holstein-Friesian cows were superovulated between days 8 and 12 of the oestrous cycle with eight doses of 480–560 IU (25–30 mg) per animal of FSH (Folicotropin, Spofa, Czech Republic) administered at 12-h intervals. Oestrus was induced by double prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) treatment (cloprostenol, Oestrophan, Spofa, Czech Republic) together with the fifth and sixth FSH injections. Three

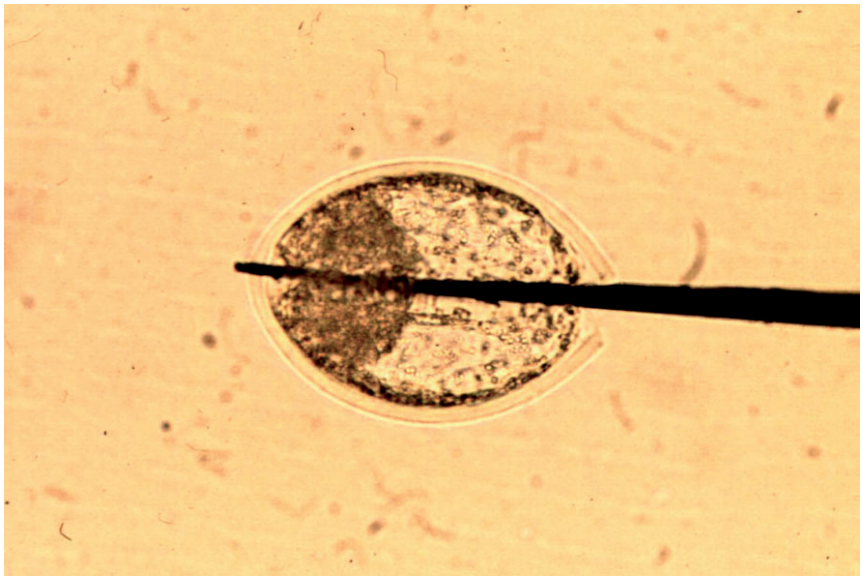

Figure 1. Bovine embryo splitting

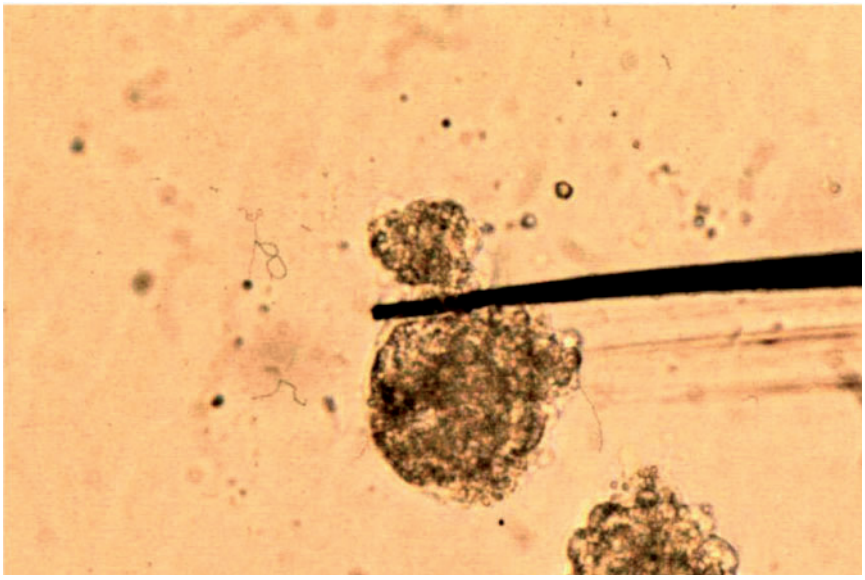

Figure 2. Biopsy of embryonic cells from a demi-embryo

artificial inseminations were performed in 48, 60 and 72 h after the first application of $PGF_{2\alpha}$.

Embryo collection and scoring

Embryos were flushed with PBS (Dulbecco's Phosphate Buffered Saline, Live Technologies, Ltd., U.K.) + 1% FCS (foetal calf serum, University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic) on Day 7 after the first insemination (D_0). Flushing as well as embryo isolation was carried out as described by Holy et al. (1990). Obtained embryos were washed with PBS + 10% FCS and classified according to their developmental stage and quality with respect to internationally accepted criteria. Only high-quality compacted morulae and early blastocysts (n = 223) were selected for microsurgical procedures. The designation of high quality was assigned to morphologically excellent (perfect embryo for its age) or good (trivial imperfections such as oval *zona pellucida,* a few, small excluded cells, or slightly asymmetrical shape) embryos.

Embryo splitting and biopsy

Microsurgical intervention was carried out with the Twinner system (AB Technology, Inc., Pullman, WA). The embryos were split with a single vertical movement of the microsurgical blade (Figure 1).

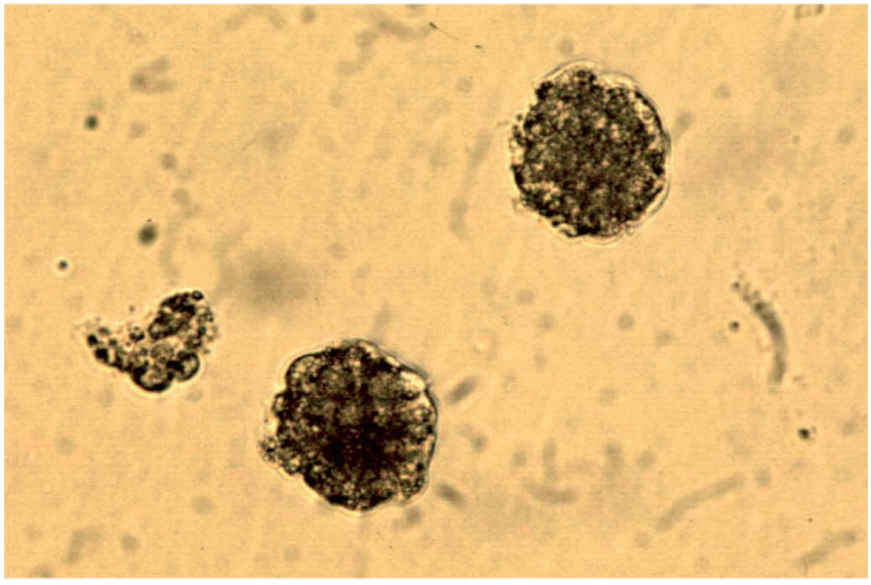

Figure 3. The clump of cells destined for sexing together with both demi-embryos

From one demi-embryo a few embryonic cells (> 5) were removed (Figure 2) by careful incision in the same way. In the case of unequal splitting, the larger demi-embryo was biopsied. Compacted morulae were bisected and biopsied in any plane. Inner cell masses as well as trophectoderms were halved in blastocysts and the trophectoderm cells were biopsied. Splitting Plus Medium (AB Technology, Inc., Pullman, WA) or PBS without proteins was used for the immobilization of embryos, no holding pipette was applied. The isolated embryonic cells (Figure 3) were analysed immediately. During the analysis, the identical pairs of demi-embryos were cultured in microdrops (100 µl) of PBS with 10% of FCS under mineral oil (Sigma-Aldrich, Inc., USA) at room temperature (20–25°C).

Sex determination

The harvested cells were immediately transferred to microtubes containing Cresol red (*o*-cresolsulphonephthalein). Embryo sexing was performed with a commercial polymerase chain reaction (PCR) kit using primers specific to the Y-chromosome determinant (YCD) according to the manufacturer's instructions (Herr et al., 1995). The PCR product was detected by UV light in agarose gel with ethidium bromide and the embryos were scored as Y-chromosome determinant positive (male) or Y-chromosome determinant negative. All instruments represent a transferable unit that can be used in field conditions (AB Technology, Inc., Pullman, WA).

Following the completion of the procedure (about 3–3.5 h after cell sample isolation), the demi-embryos were transferred. The results of PCR in morulae and early blastocysts were compared.

Embryo transfer

After sex diagnosis, the female demi-embryos were transferred into synchronized recipient heifers (n = 128, body weight 340–370 kg) with the physiological status of reproductive organs. ET was realized in pairs (bilateral, one demi-embryo into one uterus horn) or individual (ipsilateral, one demi-embryo into one recipient). Pregnancy diagnosis was performed ultrasonographically on day 21 after transfer and confirmed by rectal palpation 21 days later. In the case of bilateral transfer the number of foetuses was summarized.

Statistical analysis

The obtained data were analysed by χ^2-test (2 × 2 contingency tables). The results of the analyses are summarized in Tables 1–3.

Table 1. Sex determination in bisected D_7 bovine embryos of different stage

Stage of development	Bisected embryos (n)	Survival of identical pairs		Completed sex determination		Female sex confirmed	
		n	%	n	%	n	%
Morula	100	100	100	92	92[a]	39	42.4[c]
Early blastocyst	123	123	100	110	89.4[b]	48	43.6[d]

a:b, c:d = $P > 0.05$

Table 2. Pregnancy rate achieved after bilateral and ipsilateral ET of bisected D_7 bovine female embryos

Mode of transfer	Female identical pairs transferred (n)	Recipients used (n)	Pregnant animals		Identical twin production	
			n	%	n	%
Bilateral	46	46	26	56.5[a]	14	30.4[c]
Ipsilateral	41	82	40	48.8[b]	8	19.5[d]

a:b, c:d = $P > 0.05$

Table 3. The ratio of foetuses achieved after bilateral and ipsilateral ET of bisected D_7 bovine female embryos to original embryos

Mode of transfer	Pairs of female identical embryos transferred (*n*)	Foetuses (*n*)	Foetuses/original embryos (%)
Bilateral	46	40	87[a]
Ipsilateral	41	40	97.6[b]

a:b = $P > 0.05$

RESULTS

Table 1 summarizes the feasibility of sex determination with respect to the stage of development of bisected embryos. A total of 100 morulae were split and biopsied and the sex analysis was successfully carried out in 92 cases (92%). The female sex was confirmed in 39 embryos (42.4%). Similarly, the analysis of 123 bisected early blastocysts was successfully completed in 110 instances (89.4%) and 48 of them (43.6%) were female.

Pregnancy rates according to the mode of transfer (ET) of sexed female demi-embryos are shown in Table 2. After the bilateral transfer of 46 identical pairs of demi-embryos with determined sex to 46 recipients, 56.5% of animals became pregnant (26/46). The conception rate (48.8%) achieved after the ipsilateral ET of 41 sexed identical pairs of demi-embryos transferred into 82 recipients (40/82) was not significantly different. The pregnancy of identical twins was confirmed in 30.4% (14) and in 19.5% (8) when bilateral and ipsilateral ETs were performed, respectively. When the number of foetuses obtained after bilateral and ipsilateral transfer was related to the number of original embryos (Table 3), 87 and 97.6% of embryos conceived, respectively.

These data indicate that ET of female demi-embryos, either in bilateral or ipsilateral mode, is a very efficient approach for the achievement of a high number of heifer calves.

DISCUSSION

The method of sex determination in bovine embryos based on the amplification of specific DNA sequences of Y-chromosome using PCR provides a useful tool to influence the birth of male and female calves. The introduction of this method is a good example of the rapid application of molecular biology methods in agriculture, particularly in ET technology. Embryo biopsy can be used simultaneously for multiplex genotyping of bovine embryos (Peippo et al., 2007) and for genetic testing for hereditary diseases (Ipate et al., 2007).

Corresponding to our work, the same protocol and kit (YCD sexing kits, AB Technology, Ltd.) was used by Yu et al. (2006). The authors found that the larger number of cells in biopsies significantly reduced the incidence of samples with no DNA amplification. However, the increased sample size may affect the embryo survival, resulting in a decrease of the pregnancy rate after ET. Li et al. (2006) observed the reduced pregnancy rates (41.8%) in embryos used for biopsy and subsequently exposed to freezing and thawing procedures. Shea (1999) isolated for analysis 10 to 20% of embryonic mass and reached the satisfactory results both in sex determination rate and in pregnancy rate. Some authors (King et al., 1992; Sparks et al., 1994; Tominaga et al., 1996; Park et al., 2001) used one half of split embryo for PCR. On the other hand, only 3 to 6 cells are used as a sufficient number (Taneja et al., 1998; Park et al., 2001; Wu et al., 2004) for sex determination. In our previous experiments (Lopatarova et al., 2007) we isolated six to 12 cells from high-quality compacted morulae or blastocysts and the sex status was determined in nearly 90% of embryos. Because this number of cells seems to be sufficient for the safe sex analysis, we used a similar cell number obtained from one half of demi-embryos in this study. The sex was determined in 92% of split morulae and in 89.4% of early blastocysts (Table 1). Our results are similar to those published by other authors (85–95%) using the whole (Thibier and Nibart, 1995; Shea, 1999; Li et al., 2006; Yu et al., 2006) and bisected embryos (Lopes et al., 2001) from superovulated donors as well IVP embryos (Lopes et al., 2001; Hasler et al., 2002).

In the present study we used a similar micromanipulation unit like Thibier and Nibart (1995), Shea (1999), Lopes et al. (2001), Hirayama et al.

(2004), Tominaga and Hamada (2004), Li et al. (2006) and Yu et al. (2006). All morulae and early blastocysts survived the splitting and biopsy procedure (Table 1). We applied the quick micro-dissection method using one micromanipulator only. Shea (1999) used three methods for the biopsy of whole embryos but no differences were found between microsurgical blade, aspiration and fine glass needle in completion of sex determination and in pregnancy rates. The careful blastomere aspiration procedure for IVP embryos was introduced by Lopes et al. (2001) using two micromanipulator units. Ekici et al. (2006) performed successful biopsies after the stabilization of bovine good-quality morulae by scratching the bottom of the Petri dish with a microsurgical blade without using a micromanipulator. Ushijima et al. (1995) produced offspring by splitting D_7 blastocysts to three parts, two parts were transferred and one part was used for sex determination. King et al. (1992) cultured split morulae for 24–44 h, one part of demi-embryos was sexed and the other part was cryopreserved. They found that male embryos better resisted to manipulation and cultivation (70% versus 30%). Their findings were supported by similar results of Gutierrez-Adan et al. (2001). Live imaging experiments show that the development of female early-stage embryos progresses faster than that of male ones (Peippo et al., 2001) and more male embryos reached the morula or blastocyst stage in a glucose-supplemented medium. The recent observation of Kimura et al. (2008) suggests that the medium supplementation with glucosamine also skewed the sex ratio for males. But the effect of glucosamine was observed only after maternal-zygotic transition (after eight-cell stage).

The sex ratio of embryos can also be affected by the modification of IVF (*in vitro* fertilization) techniques. Iwata et al. (2008) discovered that the exposure of sperm to cumulus cells, either before the fertilization of denuded oocytes or during the process of fertilization of COC (cumulus-oocyte complex), increased the proportion of male embryos produced by IVF. It was hypothesized that this might be due to the capacitation state of the sperm, the cumulus-sperm interaction, and/or the ability of the sperm to bind to cumulus cells or oocytes.

In this study we confirmed the female sex in morulae (42.4%) and early blastocysts (43.6%, Table 1). Shea (1999) reached a similar rate of female sex (46–48%) in all stages of D_7–$D_{7.5}$ embryos. In our previous observations using whole embryos (Lopatarova, 2003) females slightly prevailed only in expanded blastocysts (51.2%). Carbonneau et al. (1999) confirmed the female sex in early blastocysts, blastocysts and expanded blastocysts in 50.2, 53.3 and 57.9%, respectively. Hasler et al. (2002) even showed that the sex ratio of expanded blastocysts was significantly skewed in favour of female embryos (60.3%). In our study, the identical pairs of demi-embryos were cultured *in vitro* during the sex analysis, up to 3.5 h before ET. All demi-embryos were viable after culture and could be transferred to the recipients (Table 2). Pregnancy rates (48.8 and 56.5%) were comparable to those achieved with the whole sexed or intact embryos in the routine ET schedule. Similar results (55% of pregnant recipients) were obtained by Lopes et al. (2001) when they placed the demi-embryos after bisection and biopsy into holding medium only for a few minutes and transferred them as soon as possible to recipients (within 1 to 3 h after recovery). Ushijima et al. (1995) sexed one third of embryos and transferred the other two parts to recipients after 20 h cultivation with 54% conception. A significant decrease in pregnancy rate was observed with embryos held *in vitro* for a longer period of time (> 5 h), suggesting a detrimental effect of *in vitro* conditions on embryo survival (Li et al., 2006). We support their results that cultivation conditions play a significant role in this technique.

Tominaga et al. (1996) found that female demi-embryos regenerate more progressively than male embryos. The 24 h culture of sexed demi-embryos improved the morphological quality of female demi-embryos. We observed a higher conception rate in the bilateral ET of demi-embryos in comparison with ipsilateral ET (56.5% versus 48.8%, see Table 2). A higher but statistically insignificant pregnancy rate was achieved in identical pairs with bilateral ET to one recipient 30.4% versus individual transfer to two recipients 19.5%.

Bredbacka et al. (1994) and Lopes et al. (2001) reported a similar pregnancy rate between the ipsilateral and bilateral transfer of demi-embryos. Some authors found out a lower pregnancy rate after the transfer of single halves compared to paired halves (Ozil et al., 1982; Lambeth et al., 1983), but better results were also obtained using the single demi-embryo transfer (Bredbacka et al., 1994). Ushijima et al. (1995) divided an embryo (D_7) into three parts with conception rate 54% with no difference in the case of ET of demi-embryos.

We observed no difference in pregnancy rate after the transfer of female split morula and early blastocyst, similarly like Hasler et al. (2002) and Li et al. (2006), who compared four different developmental stages after biopsy (morula, early blastocyst, blastocyst and expanded blastocyst). Only Bredbacka et al. (1994) showed that the blastocysts were more sensitive than morulae to two consecutive microsurgical treatments.

It is generally accepted that high-quality embryos are the most suitable for sex determination. However, the flushings from superovulated donors usually contain embryos of various categories and often, after evaluation under the stereomicroscope, only a limited number of embryos meets criteria typical of high-quality embryos. The use of lower-quality embryos is also possible by the isolation of blastomeres extruded into the perivitelline space (Yu at al., 2006; Lopatarova et al., 2007). This approach enlarges the spectrum of embryos which could be employed for the successful sex determination. However, the completed sex determination is lower. The splitting of excellent or good embryos with following biopsy is another possibility of increasing the pregnancy rates of desired sex from one superovulation. We reached 87% and 97.6% of foetuses per original sexed embryo (Table 3) from bilateral and ipsilateral ET, respectively. Commercial ET programs using the splitting technology have reported pregnancy rates ranging from 100 to 113% after single and 84% after double demi-embryo transfer to each recipient (Lopes et al., 2001).

In conclusion, our results document that the well established microsurgical embryo bisection, gentle isolation of the lowest number of blastomeres and excellently mastered PCR method provide a high number of reliably sexed and viable demi-embryos which efficiently implant after either bilateral or ipsilateral ET. The described method allows to produce a high number of female calves and to improve in this way the genetic potential of cattle herds in a short time interval.

REFERENCES

Avery B., Bak A., Schmidt M. (1989): Different cleavage rates and sex determination in bovine embryos. Theriogenology, 32, 139–147.

Bondioli K.R., Ellis S.B., Pryor J.H., Williams M.W., Harpold M.M. (1989): The use of male-specific chromosomal DNA fragments to determine the sex of bovine preimplantation embryos. Theriogenology, 31, 95–104.

Booman P., Kruijt L., Veerhuis R., Hengst A.M., Tiemann M., Ruch F.E. (1989): Sexing bovine embryos with monoclonal antibodies against the H-Y antigen. Livestock Production Science (Netherlands), 23, 1–16.

Bredbacka P., Vemala R., Peippo J., Bredbacka K. (1994): Survival of biopsied and sexed bovine demi-embryos. Theriogenology, 41, 1023–1031.

Carbonneau G., Twagiramungu H., Morin N., Brisson C., Durocher J., Bouquet D. (1999): Factors affecting the sex ratio of in-vitro and in-vivo produced bovine embryo. Theriogenology, 51, 396.

Edwards R.G., Gardner R.L. (1967): Sexing of live rabbit blastocyst. Nature, 214, 576–577.

Ekici H., Turan N., Sontas B.H., Helps C.R., Senunver A., Ylmaz H. (2006): Sex determination of bovine embryos using polymerase chain reaction (PCR). Revue de Medicine Veterinaire, 157, 441–444.

Galli C., Duchi R., Crotti G., Turini P., Ponderato N., Colleoni S., Lagut I., Lazzari G. (2003): Bovine embryo technologies, Theriogenology, 59, 599–616.

Gutierrez-Adan A., Lonergan P., Rizos D., Ward F.A., Boland M.P., Pintado B., de la Fuente J. (2001): Effect of the *in vitro* culture system on the kinetics of blastocyst development and sex ratio of bovine embryos. Theriogenology, 55, 1117–1126.

Han Y., Yoo O., Lee K. (1993): Sex determination in single mouse blastomeres by polymerase chain reaction. Journal of Assisted Reproduction and Genetics, 10, 151.

Handyside A.H., Kontogiann E.H., Hardy K., Winston R.M.L. (1990): Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. Nature, 344, 768–770.

Hasler J. F., Cardey E., Stokes J. E., Bredbacka P. (2002): Nonelectrophoretic PCR-sexing of bovine embryos in a commercial environment. Theriogenology, 58, 1457–1469.

Herr C.M., Holt N.A., Matthaei K.I., Reed K.C. (1990): Sex of progeny from bovine embryos sexed with a rapid Y-chromosome-detection assay. Theriogenology, 33, 247.

Herr C.M., Steel T., Lascelles A., Reed K.C. (1995): Instruction Manual: Splitting, Biopsy & Rapid Sexing of Cattle Embryos. AB Technology, Inc., Pullmann, WA, 58 pp.

Hirayama H., Kageyama S., Moriyasu S., Sawai K., Onoe S., Takahashi Y., Katagiri S., Toen K., Watanabe K., Notomi T., Yamashina H., Matzuzaki S., Minamihashi A. (2004): Rapid sexing of bovine preimplantation

embryo using loop-mediated isothermal amplification. Theriogenology, 62, 887–896.

Holy L., Lopatarova M., Krontorad P., Holy J. (1990): Systematic use of embryo transfer in cattle under condition of high animal concentration (in German). Monatsheft Veterinaermedizin, 45, 409–413.

Ipate I., Bogdan A.T., Toba L.G., Toba G.F., Ivana S. (2007): Research regarding the embryo biopsy for sexing and genetic testing the bovines embryos. Revista Romana de Medicina Veterinara, 17, 213–218.

Iwata H., Shiono H., Kon Y., Matsubara K., Kimura K., Kuwayama T., Monji Y. (2008): Effects of modification of *in vitro* fertilization techniques on the sex ratio of the resultant bovine embryo. Animal Reproduction Science, 105, 234–244.

Katska-Ksiazkiewicz L., Bochenek M., Rynska B. (2005): Effect of quality of sperm chromatin structure on *in vitro* production of cattle embryos. Archiv fur Tierzucht, 48, 32–39.

Katska-Ksiazkiewicz L., Rynska B., Bochenek M., Opiela J., Jurkiewicz J. (2006): *In vitro* production of bovine embryos using flow-cytometrically sexed sperm. Archiv fur Tierzucht, 49, 133–140.

Kimura K., Iwata H., Thompson J.G. (2008): The effect of glucosamine concentration on the development and sex ratio of bovine embryo. Animal Reproduction Science, 103, 228–238.

King W.A., Picard L., Bosquet D., Goff A.K. (1992): Sex-dependent loss of bisected bovine morulae after culture and freezing. Journal of Reproduction and Fertility, 96: 453–459.

Kobayashi J., Sekimoto A., Uchida H., Wada T., Sasaki K., Sasada H., Umezu M., Sato E. (1998): Rapid detection of male-specific DNA sequence in bovine embryos using fluorescence *in situ* hybridization. Molecular Reproduction and Development, 51, 390–394.

Lambeth V.A., Looney C.R., Voelkel S.A., Jackson D.A., Hill K.G., Godke R.A. (1983): Microsurgery on bovine embryos at the morula stages to produce monozygotic twin calves. Theriogenology, 20, 85–95.

Leonhard M., Korszenbaum M., Cotinot C., Chesne P., Heyman Y., Stinnakre M.G., Bishop C., Delouis C., Vaiman M., Fellous M. (1987): Sexing bovine embryos using Y chromosome specific DNA probe. Theriogenology, 27, 248.

Li S., Yu W., Fu J., Bai Y., Jin F., Shangguan B. (2006): Factors influencing pregnancy rates following transfer of bovine *in vivo* embryos biopsied for sex determination. Reproduction, Fertility and Development, 19, 297.

Lopatarova M. (2003): Micromanipulation methods on bovine embryos and their biotechnological application (in Czech). [Habilitation Thesis.] University of Veterinary and Pharmaceutical Sciences, Brno, 180 pp.

Lopatarova M., Krontorad P., Holy L., Zajic J. (2006): Determining the sex of early bovine embryo in practical conditions (in Czech). Veterinarstvi, 56, 696–699.

Lopatarova M., Cech S., Krontorad P., Holy L.,Hlavicova J., Dolezel R. (2007): Lower quality bovine embryos may be successfully used for sex determination. Veterinarni Medicina, 52, 540–546.

Lopes R.F.F., Forell F., Oliveira A.T.D., Rodrigues J.L. (2001): Splitting and biopsy for bovine embryo sexing under field conditions. Theriogenology, 56, 1383–1392.

Monk M., Handyside A.H. (1988): Sexing of preimplantation mouse embryos by measurement of X-linked gene dosage in a single blastomere. Journal of Reproduction and Fertility, 82, 365–368.

Ozil J.P., Heyman Y., Renard J.P. (1982): Production of monozygotic twins by micromanipualation and cervical transfer in the cow. Veterinary Record, 110, 126–127.

Park J.H., Lee J.H., Choi K.M., Joung S.Y., Kim J.Y., Chung G.M., Jin D.I., Im K.S. (2001): Rapid sexing of preimplantation bovine embryo using consecutive and multiplex polymerase chain reaction (PCR) with biopsied single blastomere. Theriogenology, 55, 843–853.

Peippo J. Huhtinen M. Kotilainen T. (1995): Sex diagnosis of equine preimplantation embryos using the polymerase chain reaction. Theriogenology, 44, 619–627.

Peippo J., Kurkilahti M., Bredbacka P. (2001): Developmental kinetics of *in vitro* produced bovine embryos: the effect of sex, glucose and exposure to time-lapse environment. Zygote, 9, 105–113.

Peippo J., Viitala S., Virta J., Raty M., Tammiranta N., Lamminen T., Aro J., Myllymaki H., Vilkki J. (2007): Birth of correctly genotyped calves after multiplex marker detection from bovine embryo microblade biopsies. Molecular Reproduction and Development, 74, 1373–1378.

Peura T., Hyttinen J.M., Turunen M., Janne J. (1991): A reliable sex determination assay for bovine preimplantation embryos using the polymerase chain reaction. Theriogenology, 35, 547–555.

Pomp P., Good B.A., Geisert R.D., Corbin C.J., Conley A.J. (1995): Sex identification in mammals with polymerase chain reaction and its use to examine sex effect on diameter of day-10 or -11 pig embryos. Journal of Animal Science, 73, 1408–1415.

Rall W.F., Leibo S.P. (1987): Production of sexed bovine pregnancies by cytogenetic analysis of cultured demi-embryos. Theriogenology, 27, 269.

Sartori R., Souza A.H., Guenther J.N., Caraviello D.Z., Geiger L.N., Schenk J.L. (2004): Fertilization rate and

embryo quality in superovulated Holstein heifers artificially inseminated with X-sorted or unsorted sperm. Animal Reproduction, 1, 86–90.

Schenk J.L., Suh T.K. Seidel Jr. G.E. (2006): Embryo production from superovulated cattle following insemination of sexed sperm. Theriogenology, 65, 299–307.

Schroder A., Roschlau D., Giehm D., Schwerin M., Thomsen P.D. (1990): Sex determination of bovine embryos using Y-specific primers in the polymerase chain reaction. Archiv fur Tierzucht, 33, 293–299.

Shea B.F. (1999): Determining the sex of bovine embryos using polymerase chain reaction results: a six-year retrospective study. Theriogenology, 51, 841–854.

Singh E.L., Hare W.C.D. (1980): The feasibility of sexing bovine morula stage embryos prior to embryo transfer. Theriogenology, 14, 421–427.

Smorag Z., Bochenek M., Pilch J. (2005): Cattle sex regulation by separation of X and Y spermatozoa – preliminary results of field experiment in Poland. Reproduction, Fertility and Development, 17, 306.

Sparks A.E.T., Canseco R.S., Russell C.G., Johnson J.L., Page J.L., Velander W.H., McGilliard M.L., Gwazdauskas F.C. (1994): Development of bovine morulae after bisection and biopsy and assessment of DNA amplification by the polymerase chain reaction. Animal Reproduction Science, 35, 1–7.

Taneja M., Rao K.B.C.A., Gangawane S., Zawar S.G., Totey S.M. (1998): Rapid sexing of bovine preimplantation embryos using polymerase chain reaction: production of calves with predetermined sex under field conditions. Indian Journal of Experimental Biology, 36, 1201–1208.

Thibier M., Nibart M. (1995): The sexing of bovine embryos in the field. Theriogenology, 43, 71–80.

Tominaga K., Hamada Y. (2004): Efficient production of sex-identified and cryosurvived bovine in-vitro produced blastocysts. Theriogenology, 61, 1181–1191.

Tominaga K., Yoneda K., Utsumi K. (1996): Influence of "Solcoseryl" during culture on the sex-dependent repair of bovine demi-embryos. Molecular Reproduction and Development, 43, 331–335.

Ushijima H., Kato T., Sakane J., Eto T., Ogawa S. (1995): Production of identical twin calves using pairs of sex-predicted demi-embryos. Journal of Reproduction and Development, 41, 71–76.

White K.L., Lindner G.M., Anderson G.B., BonDurant R.H. (1982): Survival after transfer of "sexed" mouse embryos exposed to H-Y antisera. Theriogenology, 18, 655–662.

Williams T.J. (1986): A technique for sexing mouse embryos by a visual colorimetric assay of the X-linked enzyme, glucose 6-phosphate dehydrogenase. Theriogenology, 25, 733–739.

Wu Y.S., Liu M.J., Guo Z.Q. (2004): Sex determination of bovine embryo using consecutive and multiplex polymerase chain reaction (PCR). Journal of Xinjiang Agricultural University, 27, 29–32.

Yu W., Li S., Fang J., Sun X., Cui L., Fu J., Bai Y., Fang Y., Shangguan B. (2006): Field studies on the effectiveness of the YCD embryo sexing technique in bovine. Reproduction, Fertility and Development, 19, 299–300.

Zhang M., Lu K.H., Seidel Jr. G.E. (2005): Blastocyst development of male and female bovine embryos produced by IVF with flow cytometrically sorted sperm. Reproduction, Fertility and Development, 17, 306–307.

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