Conception rate after sex determination and cryopreservation of D7 bovine embryos

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ABSTRACT: The aim of this study was to evaluate conception rate after sex determination and subsequent freezing of Day 7 (D_7) bovine embryos and to compare it with transfer of fresh female embryos. High quality embryos obtained from superovulated donors were biopsied and the embryonic cells (5–10) were analysed by PCR using specific primers for the Y-chromosome determinant. Fresh embryos were blade biopsied (n = 172) and subsequently transferred (ET) ipsilaterally to synchronized recipients. Selected embryos (n = 112) were biopsied by cell aspiration and cryopreserved for later transfer. Sex determination was successfully completed in 91.3% of fresh group embryos (44% female) and in 87.5% of freeze-thawed group embryos (45.9% female). The achieved pregnancy rate was 56.5% after fresh transfer of sexed (female) embryos whereas intact embryos (control 1) implanted in 61.9% recipients. 95.6% (43/45) of embryos survived biopsy and cryopreservation. After transfer of freeze-thawed sexed (female) embryos, pregnancy was established in 48.8% of recipients. A similar conception rate was found in intact embryos (50.7%, control 2). The results clearly demonstrate that the microsurgical technique used and subsequent PCR sex analysis allow the rapid commercial exchange of genetic resources on the basis of fresh or frozen sex-desired embryos in embryo transfer programs.

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

Embryo transfer (ET) technology is of high importance in modern cattle breeding programs. Improvements in embryo micromanipulation techniques have led to the use of direct genetic analysis of preimplantation bovine embryos by biopsy in commercial embryo transfer programs. Implementation of embryo sex determination techniques holds great potential for genetic improvement in cattle herds and market demand satisfaction.

The techniques for sex determination of bovine embryos have evolved from karyotyping of older preimplantation embryos (Hare et al., 1976), to the current variety of widely used polymerase chain reaction (PCR) protocols. The first serious attempt to sex embryos was made by Edwards and Gardner (1967) with rabbit blastocysts according to the presence of Barr bodies. Time consuming methods were used later in farm animals with high variability of results. Employed methods include cytogenetic analysis (Singh and Hare, 1980; Rall and Leibo, 1987), immunological assays (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 base of differences in cleavage rates (Avery et al., 1989). Better results were published later using PCR amplification of specific DNA sequences to determine embryonic sex in cattle (Herr et al., 1990;

Supported by the Ministry of Education, Youth and Sports of the Czech Republic (Grant No. MSM 6215712403).

Schroder et al., 1990; Peura et al., 1991), pigs (Pomp et al., 1995), horses (Peippo et al., 1995), humans (Handyside et al., 1990) and mice (Han et al., 1993). Recently published results support the suitability of the PCR method for sex determination in cattle on the basis of its high accuracy and quick results (Thibier and Nibart, 1995; Lopes et al., 2001; Ekici et al., 2006; Yu et al., 2007).

Sperm sexing and separation into X- and Y-bearing fractions for purposes of insemination or *in vitro* production of embryos (IVP) seems to be a promising procedure in sex determination. However, recent results show that the capacitation of sorted freeze-thawed fractions of sperm and the developmental capacity of IVP embryos with desired sex is very low (Zhang et al., 2005; Katska-Ksiazkiewicz et al., 2006; Blondin et al., 2009). The pregnancy rates with low doses of sexed sperm drop about 10–40% compared to conventional doses of unsexed sperm (Andersson et al., 2006; Schenk et al., 2006, 2009; Seidel Jr., 2007; DeJarnette et al., 2009). In superovulation programs, the numbers of recovered transferable embryos were significantly higher when donors were inseminated with the unsexed sperm than with the sexed inseminates (Sartori et al., 2004; Schenk et al., 2006; Hayakawa et al., 2009).

The aim of this study is to estimate the validity of a protocol comprising biopsy, to isolate embryonic cells for sex determination of high quality embryos, freeze-thawing of sex-desired embryos and subsequent ipsilateral transfer into recipients. The implementation of freeze-thawed high quality embryos of desired sex in commercial ET programs is discussed.

MATERIAL AND METHODS

Animals and treatment

Selected donors (n = 56) were stimulated according to the protocol described by Holy et al. (1990). Briefly, Holstein-Friesian cows were superovulated between days 8–12 of the oestrous cycle with eight decreasing doses of 500 IU of FSHp and 500 IU of LHp *pro toto* (Pluset®, Calier, Spain) administered at 12-h intervals. Oestrus was induced by double prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) treatment (cloprostenol, Oestrophan®, Bioveta, Czech Republic) together with the fifth and sixth FSH injections. Three artificial inseminations were performed at 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, Biochrom AG, Germany) 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 Vigro™ Holding Plus medium (AB Technology, Inc., Pullman, WA) and classified according to their developmental stage and quality with respect to internationally accepted criteria (Robertson and Nelson, 1998). Only high quality compacted morulae, early blastocysts and blastocysts (n = 284) were selected for microsurgical procedures. The designation of high quality

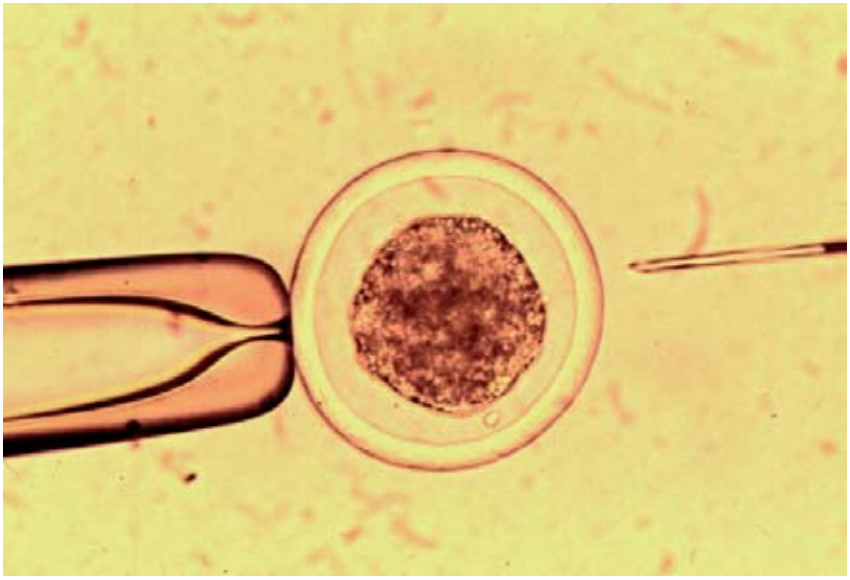

Figure 1. Preparation of pipettes and fixation of the compacted morula before biopsy by aspiration

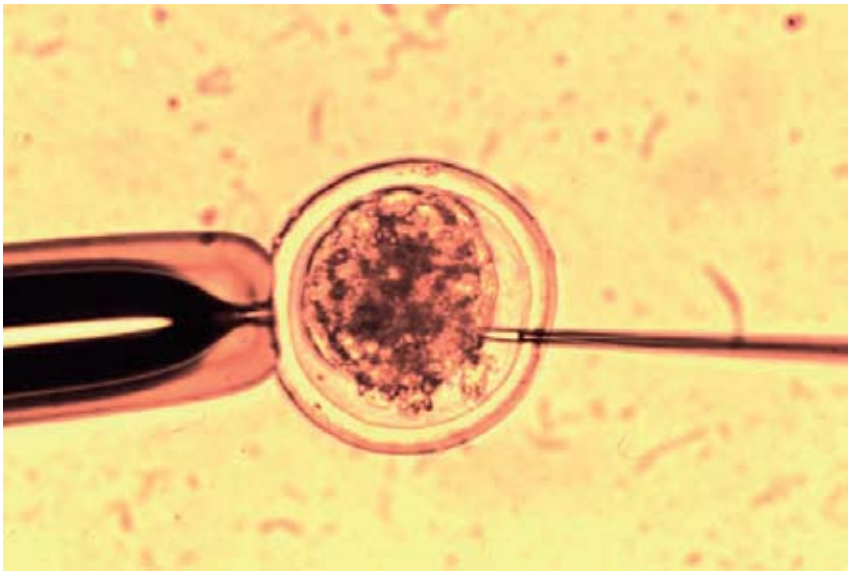

Figure 2. Aspiration of a few surface embryonic cells from the compacted morula

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 biopsy

Microsurgical intervention was carried out with the Labovert micromanipulator (Leitz, Austria). From the embryos specified for fresh transfer, a small portion of the embryo was cut off by transzonal incision using a microsurgical blade as described (Lopatarova et al., 2007). No holding pipette was used during cutting, only PBS without proteins was employed for fixation of embryos. The embryos selected for subsequent cryopreservation were transferred into fresh holding medium and immobilized using the holding pipette (outer diameter 80 µm, inner diameter 15 µm, Microtech, Czech Republic). From compacted morulae (Figures 1 and 2), a few surface embryonic cells (5–10) were removed by careful aspiration with a micro-injection pipette (outer diameter 7 µm, inner diameter 5 µm, bevel angel 50°). Care was taken to ensure that penetration of the *zona pellucida* was not accompanied by large damage. In the blastocysts, the trophectoderm cells were aspirated (5–10). The isolated embryonic cells (Figure 3) were analysed immediately. During the analysis (3.0–3.5 h), the embryos were cultured in 4-wells dishes containing 1 ml holding medium per well at ambient temperature (20–25°C).

Sex determination

The harvested cells were immediately transferred to microtubes containing Cresol red (o-Cresolsulfonephthalein). Embryo sexing was performed with a commercial polymerase chain reaction (PCR) kit using primers specific for 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 (10 mg/ml in distilled water) and the embryos were scored as Y-chromosome determinant positive (male) or Y-chromosome determinant negative (female), respectively. All instruments represent a transferable unit that can be used in field conditions (AB Technology, Inc., Pullman, WA).

Following completion of the procedure (about 3.0–3.5 h after cell sample isolation), the female embryos were fresh transferred or frozen. The results of the PCR test and sex ratio in blade biopsied embryos were compared with those that were aspiration biopsied.

Embryo freezing and thawing

The female embryos (n = 45) intended for cryopreservation were frozen using a conventional freezing method in 1.5M ethylene glycol with 0.1M sucrose (Vigro™ Ethylene Glycol Freeze Plus, AB Technology, Inc., Pullman, WA) as described by Voelkel and Hu (1992) using the HAAKE F 4Q freezer unit. The rapid thawing procedure was

Table 1. Sex determination in D_7 high quality bovine embryos before fresh and freeze-thawed ET

Mode of embryo transfer	Method of biopsy	Embryos analyzed (*n*)	Successful sex determination (*n*/%)	Female sex confirmed (*n*/%)
Fresh	blade	172	157/91.3[a]	69/44[c]
Frozen/thawed	aspiration	112	98/87.5[b]	45/45.9[d]

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

done according to Voelkel and Hu (1992) and the embryos were held for a short time in holding medium before transfer. Post-thawing embryo viability was evaluated.

Embryo transfer

After sex determination, the fresh female ($n = 69$) and freeze-thawed viable embryos ($n = 43$) were transferred ipsilaterally into synchronized recipient heifers (weight 340–370 kg). In the frame of the routine ET schedule, the intact high quality fresh ($n = 105$) and freeze-thawed embryos ($n = 75$) formed control groups (control 1 and 2) under identical conditions (during the same period on same dairy farm). Pregnancy diagnosis was performed ultrasonographically on Day 21 after transfer and confirmed by rectal palpation 7–21 days later.

Statistical analysis

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

RESULTS

Table 1 summarizes the efficiency of sex determination with respect to the method of embryonic cell biopsy. A total of 172 blade biopsied embryos were selected for fresh transfer and sex analysis was successfully carried out in 157 of them (91.3%). Female sex was confirmed in 69 embryos (44%). Similarly, the analysis of 112 embryos biopsied by aspiration and selected for freeze-thaw transfer was successfully completed in 98 instances (87.5%) and 45 of them (45.9%) were female.

Pregnancy rates of the sexed fresh embryos and the intact fresh embryos (control 1) are compared in Table 2. After the transfer of 69 female embryos, 39 animals became pregnant (56.5%). The conception rate of 61.9% after ET of intact control embryos was not significantly different (65/105). Embryo biopsy, sexing and cryopreservation did not significantly influence conception rate in comparison with the intact cryopreserved embryos (control 2, Table 3). After the thawing of 45 female embryos, 43 of them were eligible for ET (95.6%) and 48.8% of them (21) became pregnant. After ET of the control embryos (control 2), 50.7% of recipients became pregnant (38/75).

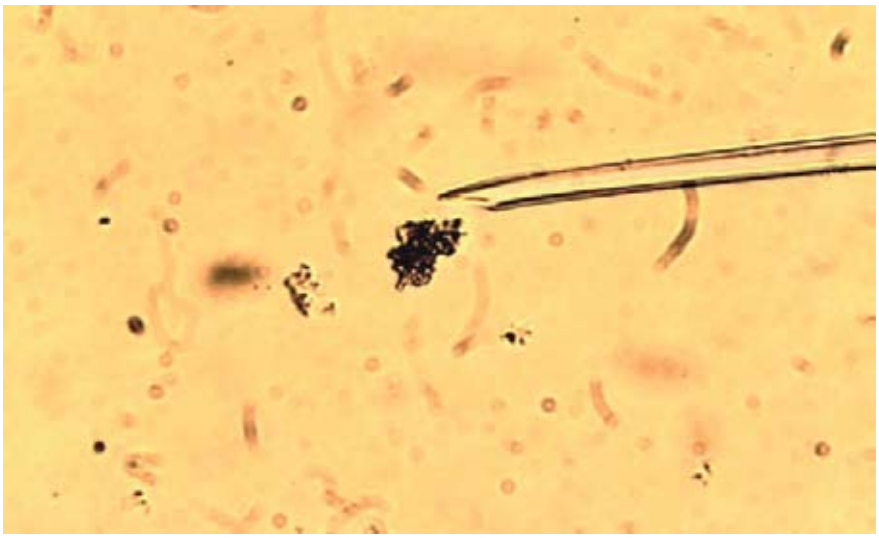

Figure 3. The clump of cells isolated during biopsy and subjected to sexing

Table 2. Pregnancy rate achieved after fresh ET of D_7 bovine sexed (female) versus unsexed (control 1) embryos

Embryo biopsy and sexing	Embryos transferred (*n*)	Pregnant animals (*n*/%)
Yes	69	39/56.5[a]
Not (control 1)	105	65/61.9[b]

a : b = $P > 0.05$

Table 3. Pregnancy rate achieved after freeze-thawed ET of D_7 bovine sexed (female) versus unsexed (control 2) embryos

Embryo biopsy and sexing	Freeze-thawed embryos (*n*)	Transferred of surviving embryos (*n*/%)	Pregnant animals (*n*/%)
Yes	45	43/95.6[a]	21/48.8[c]
Not (control 2)	75	75/100[b]	38/50.7[d]

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

These data indicate that ET of sexed-fresh and sexed-cryopreserved embryos represents a very efficient approach for achieving a high number of calves with desired sex (Figure 4).

DISCUSSION

The method of sex determination in bovine embryos using embryonic cell biopsy and DNA analysis represents a useful tool for influencing the sex of calves. The technique expands to include other DNA markers associated with valuable production traits. Nowadays it can be used for multiplex genotyping of bovine embryos (Peippo et al., 2007; Hirayama et al., 2008; Alonso et al., 2009) and for genetic testing for hereditary diseases (Hirayama et al., 2004; Ipate et al., 2007).

In our previous experiments (Lopatarova et al., 2007, 2008) for isolation of embryonic cells we used the blade biopsy for fresh embryo transfer. In this study, we have introduced the aspiration procedure to the biopsy of embryonic cells which can retain embryos with high developmental competence and minimize the damage to the *zona pellucida* before the freeze-thaw procedure. A glass holding pipette stabilizes the embryo by negative pressure while the other pipette performs the biopsy. An embry-

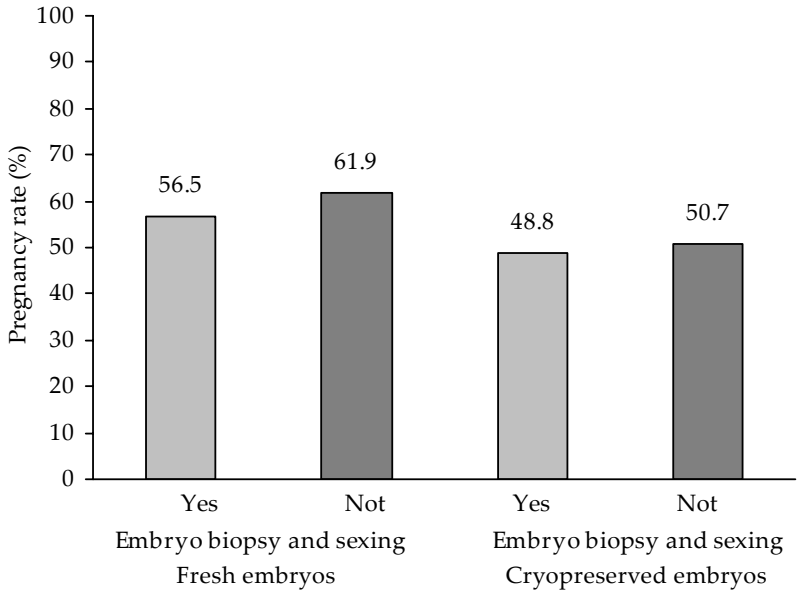

Figure 4. Pregnancy rate after sexing and cryopreservation of bovine D_7 embryos

onic cell aspiration procedure for *in vitro*-produced (IVP) embryos was applied by Lopes et al. (2001) using two micromanipulator units. Shea (1999) used three methods for biopsy of whole embryos but no differences were found between microsurgical blade, aspiration and fine glass needle in completion of sex determination (86–92%) as well as in pregnancy rates (49–60%), achieved after transfers of fresh embryos. However, frozen blade biopsied embryos, usually *zona*-free embryos, show lower (23%) pregnancy rates after transfer in comparison with needle biopsy (41%; Shea, 1999). In contrast, the present experiments demonstrated better pregnancy rates with the aspiration method and freeze-thawing of embryos (48.8%). In IVP embryos biopsied by aspiration, the mortality rate was 10.3% and the pregnancy rate was 31.7% after ET of fresh embryos (Alonso et al., 2009).

In this study, the gender was determined in 91.3% of embryos for fresh transfer and in 87.5% of embryos intended for freezing (Table 1), which accords with our previous work with whole, intact (88.7%; Lopatarova et al., 2007) and splitted embryos (89.4–92%; Lopatarova et al., 2008). Our results are similar to those published by other authors (85–95%) using embryos from superovulated donors (Thibier and Nibart, 1995; Shea, 1999; Li et al., 2007; Yu et al., 2007) as well as IVP embryos (Lopes et al., 2001; Hasler et al., 2002; Tominaga and Hamada, 2004). However, the successful sex detection was inversely related to the number of isolated embryonic cells (≤ 3 cells 85.5%, 4–6 cells 97.4%, ≥ 7 cells 100%; Lacaze et al., 2008).

In this study we confirmed female sex in 44 and 45.9% of embryos (Table 1). Shea (1999) and Lacaze et al. (2008) reported a similar rate (44-48%) in all stages of D_7–$D_{7.5}$ embryos. Hasler et al. (2002) even showed that the sex ratio of expanded blastocysts was significantly skewed in favour of female embryos (60.3%). They further proved that the overall percentage of male IVP embryos was significantly higher (53%) than the percentage of male *in vivo* produced embryos (49.2%). King et al. (1992) and Gutierrez-Adan et al. (2001) found that male embryos were more resistant to manipulation and cultivation. Nedambale et al. (2004) recovered IVP embryos from culture and then after vitrification. After warming, the embryos were cultured for 48 h and sexed by PCR. The embryos that reached the blastocyst stage earlier were predominantly males and these male embryos had better morphology and subsequently hatched at a greater rate than female embryos. PCR analysis for sexing of *in vivo* produced D_7 embryos indicated that male embryos developed faster and were graded higher than female embryos (Tominaga, 2004). The sex ratio was significantly shifted to the male IVP embryos also in more recent publications (Kimura et al., 2008; Alonso et al., 2009).

Comparison of ethylene glycol or glycerol as cryoprotectants did not show any influence on the pregnancy rate (Shea, 1999; Lopes et al., 2001). We used only 1.5M ethylene glycol for a rapid and effective thawing procedure. Hasler et al. (2002) first froze embryos in ethylene glycol or glycerol and biopsies were carried out after thawing. The freeze-thawing-biopsy protocol rendered again, with both cryoprotectants, satisfactory pregnancy rates (about 50%). Also the ultra-rapid vitrification, using the gel-loading tip (Tominaga, 2004), is acceptable for embryos that underwent the biopsy and method is available to practical use in the field.

The experiments with IVP blastocysts showed a similar post-thaw survival rate for biopsied (92.3%) and intact (96.2%) embryos after slow cooling in 0.7M glycerol plus 0.1M sucrose, respectively (Tominaga et al., 2007). In our study, 95.6% of embryos survived the biopsy, *in vitro* culture during the sex analysis (up to 3.5 hours) and freezing procedures. In the control group (control 2), all intact embryos were viable after thawing and could be transferred to the recipients (Table 3).

However, the IVP bovine embryos are more sensitive to these procedures than those derived *in vivo*, probably due to accumulation of lipids during culture (Pereira et al., 2008). These authors proved that the resistance of IVP embryos to biopsy and following vitrification was significantly improved by supplementing bovine serum-containing media with trans-10, cis-12 conjugated linoleic acid (CLA) that decreases embryo cytoplasmic lipid deposition during *in vitro* culture. Embryos cultivated in CLA (CLA embryos) show higher post-warming survival rate (95%) and expansion rate (64.6% after 24 h of culture) than embryos cultured in conventional media (62.5 and 27.5%, respectively).

The viability of fresh biopsied embryos after ET varies from 49 to 62% (Lopes et al., 2001; Hasler et al., 2002; Li et al., 2007) using the various mode of biopsy. Our result in pregnancy rate (Table 2) was comparable with published data and does not differ between the pregnancy rates after ET of fresh intact (control 1, 61.9%) and sexed embryos (56.5%).

We obtained a very similar pregnancy rate (Table 3) comparing experimental (biopsied and

frozen) embryos (48.8%) with control 2 (intact and frozen) embryos (50.7%). In contrast, Hasler et al. (2002) reported that transfer of the frozen embryos in ethylene glycol produced a lower pregnancy rate after biopsy (49.8%) than did intact embryos (65.6%). In the study of Lacaze et al. (2008), the pregnancy rate after transfer of freeze-thawed biopsied female embryos averaged 47.2% (corresponding to our study) and the result was mainly influenced by the parity of recipients (heifers 51.7% vs. cows 26.3%).

In our study, no significant differences were found between the pregnancy rates of biopsied fresh (56.5%) and freeze-thawed (48.8%) embryos. However, Shea (1999) reported a great difference between pregnancy rates after the transfer of biopsied non-frozen (58%) and in glycerol (37%), or in ethylene glycol (37%), frozen embryos. Li et al. (2007) observed reduced pregnancy rates in a large number of animals (41.8%) with embryos used for biopsy and subsequently exposed to freezing and thawing procedures, whereas the implantation of fresh embryos after biopsy did not affect pregnancy rate (49.6%) compared to non-biopsied fresh and freeze-thawed embryo groups (52.9 and 46.6%, respectively).

Comparing the efficiency of embryo sexing versus sperm sexing method, recent publications report worse results with sexed sperm compared to unsexed sperm, when X chromosome-bearing fractions of sorted sperm were used for IVF (Blondin et al., 2009), for insemination (Schenk et al., 2009) and in multiple ovulation and embryo transfer (MOET) programs (Hayakawa et al., 2009; Peippo et al., 2009). The use of low-dose X-sorted spermatozoa for the insemination of superovulated embryo donors can improve the proportion of transferable female embryos produced but this potential may be not realised in commercial bovine practice, due to reduced fertilization rates using X-sorted spermatozoa (Peippo et al., 2009). The lower effectiveness may be due to procedures associated with sorting, handling of sperm, bull selection, instrumentation, mode of insemination, management level, etc. (Garner and Seidel Jr., 2008).

In conclusion, our results document that the well established gentle isolation of the lowest number of embryonic cells by microsurgical biopsy of embryos, an efficient and accurate PCR method, and careful cryopreservation provides for a high number of reliably sexed and freeze-thawed viable embryos that successfully implant after ET (Figure 4). The described method allows, under field conditions, the production of a high number of calves of desired gender and improves the genetic potential of cattle herds in a short time interval.

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Received: 2009–11–02
Accepted after corrections: 2010–01–29

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