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Full Length Research Paper

Sexing bovine pre-implantation embryos using the polymerase chain reaction: A model for human embryo sexing

Cenariu M, Pall Emoke and Groza I*

Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania.

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The paper aims to present a bovine model for human embryo sexing. Cows were super-ovulated, artificially inseminated and embryos were recovered 7 days later. Embryo biopsy was performed; DNA was extracted from blastomeres and amplified using bovine-specific and bovine-Y-chromosome-specific primers, followed by agarose gel electrophoresis. Embryos were then transferred to synchronized recipients and PCR predicted sex was compared to the actual sex of the newborn calves. Results showed a 39% pregnancy rate and a 95.65% accuracy of the sexing method. The sexing method can also be applied to human embryos, using different primers, designed for human DNA.

Key words: sexing, embryo, PCR, bovine.

INTRODUCTION

In vitro fertilization represents nowadays a modern assisted reproductive technology that can be applied to couples with fertility problems that make natural conception impossible. In such cases, pre-implantation diagnosis represents a more convenient alternative to prenatal diagnosis, as it avoids traumatizing abortions in case of an inherited disease (Harper et al., 1994).

Sexing *in vitro* fertilized human embryos could represent a useful technique, especially for couples at risk of transmitting X-linked diseases (Handyside et al., 1989). In the early 1990s, fluorescent *in situ* hybridization (FISH) was applied as a more efficient technique to perform embryo sexing rather than using polymerase chain reaction (PCR) to amplify a Y-chromosome sequence (Harper and Sengupta, 2012). PCR has become lately a more efficient technique that can rapidly perform genetic diagnosis (Harper et al., 2010; Spits and Sermon, 2009). It has been used in many species, including cattle, in order to amplify a Y-chromosome specific DNA sequence and therefore to determine the sex of an embryo, as a supportive technology meant to increase the efficiency of embryo transfer (Garcia, 2001; Bredbacka, 2001; Hasler, 2003; Kahi and Rewe, 2008). Nevertheless, its use for human embryo sexing is still not very widely spread and many laboratories still use FISH as the golden standard for human embryo sexing (Martinhago et al., 2010). When PCR was compared with FISH for human embryo sexing, the PCR index of 74.3% was lower than the 80% index of FISH. However, there was no discrepancy in embryo diagnosis between the two techniques, which showed absolute specificity (Martinhago et al., 2010).

The experience accumulated in veterinary reproductive biotechnologies recommends PCR as being an easier, faster and cheaper method of embryo sexing as compared to FISH. The cow can represent a good model for human embryo sexing as bovine embryos are easy to obtain and there is no ethical issue related to their use for research. Furthermore, the only adaptation to the technique would be another set of primers targeted to human DNA, while the general principles and procedures can remain the same. The purpose of this paper was to present a bovine model for human embryo sexing and to

*Corresponding author. E-mail: ioangroza55@yahoo.com. Tel: +40-264-596384 Ext.163. Fax: +40-264-593792.

Abbreviations: PCR, Polymerase chain reaction; **FISH,** fluorescent *in situ* hybridization; **IU,** international units; **FSH,** follicle stimulating hormone; **LH,** luteinizing hormone; **BSA,** bovine serum albumin; **IETS,** international embryo transfer society; **UV,** ultraviolet.

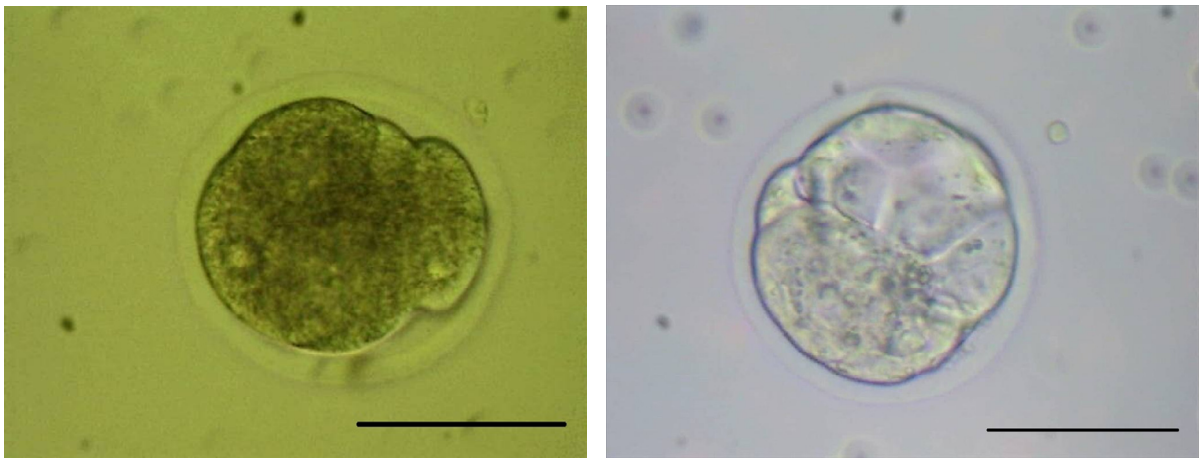

Figure 1. Bovine morula (left) and blastocyst (right); bar = 100 µm.

propose an accurate technique that could be easily adapted in human assisted reproduction laboratories.

MATERIALS AND METHODS

The research was carried out on a batch of ten Holstein-Friesian cows that were superovulated using a total dose of 1000 IU porcine FSH-LH (Pluset, Carlier). The medication was administered via intramuscular injection as follows:

Day 1*: 08.00 h - 3 ml (150 UI FSH + 150 UI LH);
20.00 h - 3 ml (150 UI FSH + 150 UI LH).
Day 2: 08.00 h - 2.5 ml (125 UI FSH + 125 UI LH);
20.00 h - 2.5 ml (125 UI FSH + 125 UI LH).
Day 3: 08.00 h - 2 ml (100 UI FSH + 100 UI LH);
20.00 h - 2 ml (100 UI FSH + 100 UI LH);
- 2 ml Cloprostenol (PGF Veyx Forte, Veyx-Pharma GmbH).
Day 4: 08.00 h - 1.5 ml (75 UI FSH + 75 UI LH);
20.00 h - 1.5 ml (75 UI FSH + 75 UI LH).
Day 5: 08.00 h - 1 ml (50 UI FSH + 50 UI LH);
20.00 h - 1 ml (50 UI FSH + 50 UI LH).
*Day 1 corresponded to day 11 of the sexual cycle

The cows were then artificially inseminated three times, at 12, 24 and 36 h from the onset of estrus, which usually occurred 36 to 48 h from the last Pluset administration. The embryos were recovered at 6.5 to 7.5 days after the last insemination, using a DISSI CH18 catheter with Luer adaptor (Minitub). The flushing liquid consisted of the BoviPro medium with BSA (Minitub) that was maintained at 37°C. Embryo identification was performed using a stereo microscope and each embryo was then passed into a Petri dish with BoviPro holding medium (Minitub) for morphologic evaluation using a microscope. Embryo quality was graded according to the guidelines suggested by the international embryo transfer society (IETS). The embryos that had reached the morula or blastocyst stage (Figure 1), and were classified as Grade 1 (excellent or good) were considered suitable for embryo biopsy, in order to collect the blastomeres needed for embryo sexing. They had symmetrical and spherical shape, with individual blastomeres that were uniform in size, colour and density, with minor irregularities and intact embryonic mass, smooth zona pellucida, without any concave or flat surfaces. The biopsies were carried out using an Olympus Narishige ONO-131 three axis hydraulic micromanipulator. The biopsy medium consisted of PBS supplemented with 0.3% protein and the embryo was stabilized using the scratch bottom technique. A microblade was placed on top of the embryo, close to one of the edges, and moved back and forth until a small part of the embryo was literally sliced away. The biopsy usually represented approximately 25 to 30% of the total embryonic cell mass.

After biopsy, the embryos were immediately transferred to recipient cows, while the blastomeres were submitted to DNA extraction using the commercial Isolate Genomic DNA Mini Kit (Bioline), whose core reagent is proteinase K, following the instructions in the product manual.

Duplex PCR was used for DNA amplification, using two sets of primers, as follows: The first set was obtained using a bovine specific DNA sequence (1715 bovine satellite DNA) in order to show the presence of DNA in all samples. Thus, the sequence of the two primers was: Upstream 5' – TGG AAG CAA AGA ACC CCG CT – 3' downstream: 5' – TCG TGA GAA ACC GCA CAC TG – 3' (Peura et al., 1991); the second set of primers was obtained using the BRY4a repetitive sequence from the bovine genome that is highly specific for the Y chromosome and is present only in males. The sequence of the primers was upstream: 5' – CTC AGC AAA GCA CAC CAG AC – 3' and downstream: 5' – GAA CTT TCA AGC AGC TGA GGC – 3' (Peura et al., 1991).

DNA quantification was made using a Nanodrop spectrophotometer, while the PCR mixture consisted of 10 ng DNA, 20 pmol of each primer and 45 µl of MyTaq Red Master Mix (Bioline). The amplification of the DNA sequences was made using the following amplification scheme: Sample heating at 96°C for 3 min, 33 cycles of denaturation at 95°C for 1 min, primer annealing at 58°C for 1 min, primer extension at 72°C for 1 min and the final extension at 72°C for 5 min. Electrophoresis of the amplified samples was performed in a 2.5% agarose gel followed by examination using a UV transilluminator. The presence of a single 216 bp band corresponding to the bovine specific primers suggested the absence of Y-specific DNA sequences and thus the sample was considered to come from a female embryo. The presence of two bands, one for the bovine specific primers (216 bp) and another for the Y-chromosome specific primers (301 bp) confirmed the presence of a Y-specific DNA sequence and thus the sample was considered to come from a male embryo.

The pregnancies obtained after the transfer of biopsied and sexed embryos were evaluated by ultrasonography, performed 30 days after the transfer, and the pregnancy rate was calculated based on the number of inseminated versus pregnant cows. The accuracy of embryo sexing was evaluated at birth, when the

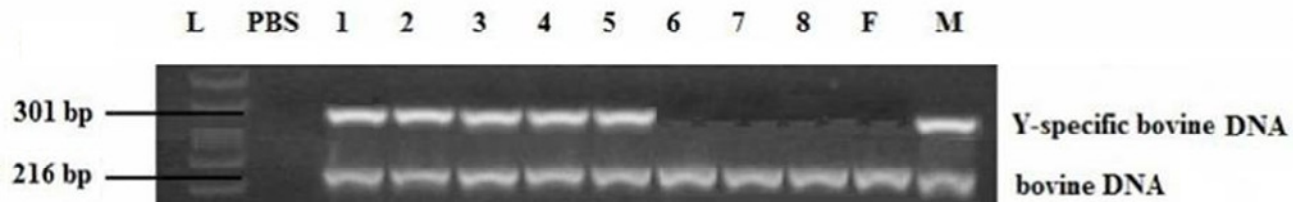

Figure 2. DNA bands obtained for 8 of the samples (L = ladder, PBS = negative control, 1-8 = DNA samples, F = female control, M = male control).

predicted sex was compared with the morphological sex of the newborn.

RESULTS AND DISCUSSION

The superovulation treatment had a positive response in all cows, the rectal palpation performed before embryo recovery showing a high number of corpora lutea on each ovary. Following embryo recovery and identification; a total number of 63 embryos were obtained from the 10 donor cows, meaning an average of 6.3 embryos/donor. The morphologic evaluation performed using a stereomicroscope pointed out the stage of development reached by each embryo as well as their degree of structural integrity. A total number of 51 intact morulae and 8 intact blastocysts were obtained, while 4 of the embryos were degenerated. The 59 intact embryos were further used for biopsy, the blastomeres being collected in good conditions.

Following DNA extraction, amplification and electrophoresis of the 59 samples, the following results have been obtained: 37 samples showed a single 216 bp DNA band and thus were considered to come from female embryos; 22 samples presented both a 216 bp DNA band and a 301 bp DNA band and thus were considered to come from male embryos (Figure 2).

The pregnancy rate in recipient cows was of 39%, 23 of 59 females being diagnosed as pregnant. At birth, one of the 23 calves presented a different sex than the predicted one (male instead of female) which means a 95.65% accuracy of the PCR sexing method. A critical analysis of the results pointed out various aspects that are worthwhile mentioning. First of all, the pregnancy rate of 39% obtained after transferring the biopsied embryos to recipient cows was slightly lower than the one reported by other authors. Following the transfer of nineteen sexed morulae into recipient females on the seventh day after fertilization, Machaty et al. (1993) obtained a 52.6% pregnancy rate, while Shea (1999) obtained a 58% pregnancy rate after transferring sexed embryos. Lopes et al. (2001) obtained comparable pregnancy rates for bisected (60%) and intact embryos (61%), while Lopatarova et al. (2008) showed a 56.5% pregnancy rate after the transfer of sexed demi-embryos. Akiyama et al. (2010) showed that the pregnancy rate after transferring vitrified sexed bovine embryos was of 38.7% and 34.8%. The biopsy technique used in our study involved the aspiration of several blastomeres (25 to 30% of the inner cell mass), as it was performed by slicing the embryo with a microblade. Therefore, the large size of the biopsy could have been the reason for a decreased pregnancy rate. Although other studies (Park et al., 2000; Chrenek et al., 2001; Carneiro et al., 2011) have shown that the biopsy of a single blastomere is enough in order to perform sexing and other genotype analysis on embryos, an accuracy of sex prediction of 100% has been reported when the number of blastomeres dissociated from a morula exceeded more than three (Zoheir and Allam, 2010). Therefore, equilibrium has to be found between removing a large number of blastomeres and damaging the embryo (therefore reducing the pregnancy rate) and removing a single blastomere and risking decreasing the accuracy of the method.

The accuracy of the method (95.65%) is quite high, being comparable with the one obtained when fluorescent *in situ* hybridization (FISH) was used to determine the sex of bovine embryos (96% reported by Lee et al. (2004). The advantages of the PCR method over the FISH method would be the reduced amount of time needed for an analysis as well as the reduced costs involved.

Another modern approach to determine and even to influence the sex of the offspring would be to perform artificial insemination or *in vitro* fertilization using sexed sperm. This biotechnology is widely used in cattle and starts to gain interest in human medicine, as more than a hundred babies are born annually from use of sexed human sperm by flow cytometry/cell sorting. However, this technology is still rather expensive, and fertility of sexed sperm is lower than unsexed controls in most instances (Seidel, 2009).

While most of the earlier approaches used in order to sex human embryos referred to fluorescent *in situ* hybridization (Penketh et al., 1989, Harper et al., 1989, Mori and Shiota, 1994), the recent studies focus on PCR as a very accurate tool of preimplantation diagnosis, including sex determination, in humans (Martinhago et al., 2010). Therefore, we consider that bovine embryo sexing using PCR can represent a successful animal model for human embryo sexing, using adapted primers, designed for human DNA and taking into consideration

all ethical aspects.

Conclusion

The most important issue with embryo sexing remains the biopsy method used for the removal of blastomeres needed for DNA extraction. An equilibrium has to be found between removing a large number of blastomeres and damaging the embryo (therefore reducing the pregnancy rate) and removing a single blastomere and risking decreasing the accuracy of the method. This especially true when sexing is followed by cryopreservation and not direct transfer, as freezing decreases the viability of embryos even more, and the use of a biopsy method that produces little damage to the zona pellucida becomes vital. The PCR method of sexing bovine embryos presented here could easily be adapted to human embryos, using an adapted set of primers, and could replace the FISH method that is currently more popular.

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