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ORIGINAL PAPER

Sexing of Bovine Embryos Using Polymerase Chain Reaction (PCR) and Fluorescent *In Situ* Hybridization (FISH)

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Abstract

The purpose of this paper was to evaluate the results obtained for bovine embryo sexing using the polymerase chain reaction (PCR) and fluorescent in situ hybridization (FISH) and to compare them in order to decide which of the two methods is more accurate and yields better results while necessitating less effort. We took into consideration the pregnancy rate obtained after the transfer of biopsied embryos, the percentage of correctly sexed embryos evaluated at birth (when the predicted sex was compared with the actual sex of the newborn) as well as other characteristics related to the difficulty of the method, expenses and suitability to a minimally equipped laboratory. We concluded that the polymerase chain reaction is more accurate and suitable for sexing preimplantation bovine embryos, being in the same time easier to perform than the fluorescence in situ hybridization.

Key words: embryo, cattle, sexing, PCR, FISH

Introduction

The biotechnology of embryo transfer in cattle represents a major breakthrough in bovine reproduction and is nowadays considered to be an industry that produces nearly 500,000 embryos yearly from superovulated cows, on a worldwide basis (Hasler [8]). Sexing of embryos before transplantation represents a new and useful technique that increases the efficiency of embryo transfer, enabling the choice of embryos to be transferred, according to their gender (Bredbacka [2], Cenariu *et al.* [3]). Researchers have lately tried to find the most suitable method to detect the sex of bovine embryos, which would produce as little damage to the embryo as possible but, in the same time, provide a high degree of accuracy and be easy to perform (Lee *et al.* [10], Shea [15]). A number of invasive and non-invasive methods have been imagined, depending on whether or not a biopsy of embryonic tissue is required (Garcia [5]). The vast majority of studies have shown that the most effective approach to differentiate the gender of a bovine embryo is to identify the presence or absence of a Y-chromosome specific DNA sequence found only in male embryos. The embryos in which this sequence is shown are considered to be males while the others are females (Akiyama *et al.* [1], Cenariu et al. [4], Groza [7], Lopes *et al.* [11], Taketo *et al.* [16]).

The purpose of this paper was to evaluate the results obtained for bovine embryo sexing using the polymerase chain reaction (PCR) and fluorescent *in situ* hybridization (FISH) and to compare them in order to decide which of the two methods is more accurate and yields better results while necessitating less effort.

Materials and method

A total number of 152 bovine embryos have been used for sexing, and were divided into 2 batches:

- **batch 1**, made up of 76 bovine embryos that were sexed using the **polymerase chain reaction** (PCR);
- **batch 2**, made up of 76 bovine embryos that were sexed using the **fluorescent *in situ* hybridization** (FISH).

The embryos have been non-surgically collected from a batch of 16 Simmental donor cows, following superovulation and artificial insemination. The embryos have been morphologically evaluated using a stereomicroscope and biopsy has been performed in order to collect a small number of blastomeres from the inner cell mass. After biopsy, the embryos have immediately been transferred to recipient cows, whose estrous cycle had previously been synchronized with the donor cows (Groza *et al.* [6]).

The blastomeres obtained from batch 1 were submitted to DNA isolation and identification of certain nucleotide sequences found only on the Y chromosome, using specific primers. In short, the technique consisted of the following:

a. DNA extraction from the blastomeres using proteinase K;
b. One set of primers used for PCR were obtained using a bovine specific DNA sequence (1715 bovine satellite DNA) in order to show the presence of DNA in all the 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' which yield a PCR product of 216 bp (Peura *et al.* [13]);
   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' which yield a PCR product of 301 bp (Peura *et al.* [13]).
c. Setting up of the PCR mixture that consisted of 10 ng DNA, 40 pmol of each primer and 45 μl of Platinum High Fidelity PCR Supermix (Invitrogen).
d. Amplification of the DNA sequences, using a thermocycler;
e. Electrophoresis of the amplified samples in a 1.5% agarose gel stained with Gelstar Nucleic Acid gel stain;
f. The presence of a single band of 216 bp corresponding to the bovine specific primers suggested the absence of Y-specific DNA sequences and thus made us classify the embryo as female(figure 1);
   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 made us classify the embryo as male (figure 1).

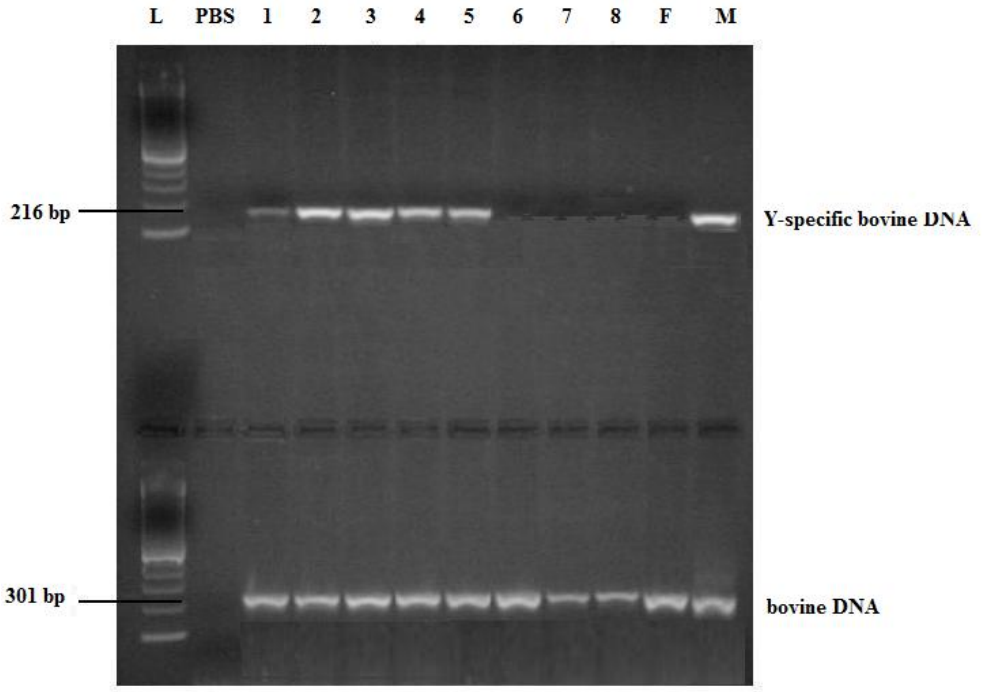

Figure 1 - Agarose gel obtained after the electrophoresis of 8 DNA samples:
1-5 represent male embryos, 6-8 represent female embryos (L = ladder, PBS = negative control, 1-8 = the DNA samples, F = female control, M = male control) The amplification scheme consisted of sample heating at 96ºC for 3 min., 33 cycles of denaturation at 95ºC for 1 min., primer annealing at 58ºC for 1 min. and primer extension at 72ºC for 1 min. and the final extension at 72ºC for 5 min.

The blastomeres obtained from batch 2 were treated with 1 µg/ml vinblastine sulphate for 6 hours in order to induce the chromosomal metaphases, fixed on a slide using methanol and acetic acid and then kept in the freezer until use (Jin *et al.* [9]).

The DNA probe was synthesized using the Y-chromosome specific BtY2 gene from which the following primers have been obtained: upstream 5' - TGT TGT GAA GAA GGT GCC CA - 3' and downstream 5' - AGT TTG AGG GTG GTT GGT CG - 3' (Lee *et al.* [10]).

The primers were used to amplify the male specific DNA sequence obtained after collecting blood from a bull and isolating the DNA from it. The amplification mixture consisted of 5 ng DNA, 1.5mM of each primer and 45 µl Platinum High Fidelity PCR Supermix (Invitrogen). The amplification conditions were: heating the mixture at 95ºC for 3 minutes followed by 33 cycles of denaturation at 95ºC for 30 seconds, primer annealing at 55ºC for 30 seconds and primer extension at 72ºC for 30 seconds. The final extension consisted of keeping the samples at 72ºC for 7 minutes. After the amplification, the samples were run on a 1 % agarose gel stained with Gelstar Nucleic Acid gel stain. Following electrophoresis, the gels were examined using a UV trans-illuminator, the bands were cut and the DNA was extracted from the gel in order to obtain the DNA probes used for fluorescent in situ hybridization. The DNA probes were biotinylated by nick translation.

The fluorescent in situ hybridization consisted of the following steps: rehydration of the blastomeres using decreasing concentrations of ethanol, target retrieval using heat and sodium citrate buffer, blastomere digestion using Triton X and proteinase K, fixation of the blastomeres using paraformaldehyde, application of the in situ frames, application of the hybridization buffer containing 1% DNA probe, hybridization reaction using a thermocycler and the following hybridization scheme: 94ºC for 6 minute and 37ºC for 16 hours, washing the slides in 3 baths of PBS.

The amplification of the fluorescent signal has been achieved using the tyramide signal amplification kit (Perkin-Elmer), which contains streptavidin-HRP, FITC conjugated tyramide and hydrogen peroxide. The slides were examined under a fluorescence microscope, in order to observe the green fluorescence in male embryos (figure 2).

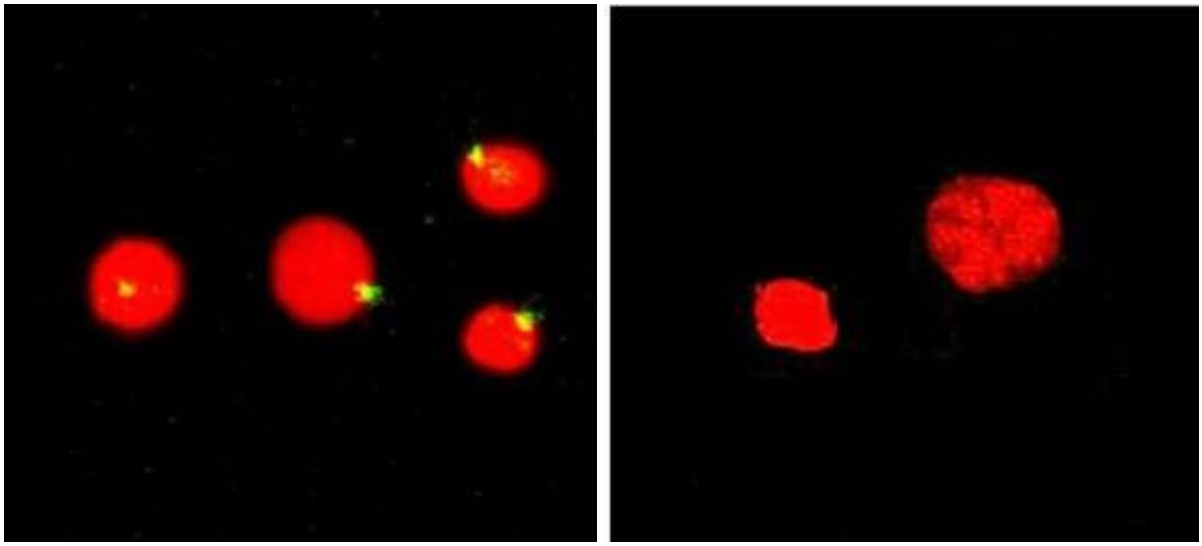

Figure 2. The fluorescent blastomeres derived from male embryos(left) and the blastomeres without fluorescence derived from female embryos (right) (Lee *et al.* [10])

The pregnancy rates obtained after the transfer of biopsied and sexed embryos were evaluated by rectal palpation, 60 days after the insemination, while the accuracy of embryo sexing was evaluated at birth, when the predicted sex was compared with the morphological sex of the newborn.

Results and discussions

The embryo biopsy was successful for all of the embryos and the blastomeres were collected in good conditions.

After performing the experiences in the blastomeres belonging to ***batch 1***, the following results have been obtained:

From the 76 embryos obtained, 70 (92.1 %) had reached the morula stage, while 6 (7.9%) were in the early blastocyst stage. In what the quality of embryos was concerned, 73 embryos (96%) were transferable and were used for embryo biopsy while 3 (4%) could not be used for this purpose.

After the amplification and gel electrophoresis of the 73 samples, the following results have been obtained:

- **34 samples** presented a single 216 bp DNA band when the bovine specific primers were used and no band when the Y-chromosome specific primers were used and thus they were considered to come from **female** embryos,

- **39 samples** presented a 216 bp DNA band when bovine specific primers were used and a 301 bp DNA band when Y-chromosome specific primers were used, thus being considered to come from **male** embryos.

When evaluating the pregnancy rate in recipient cows in which sexed embryos had been transferred, the following results have been obtained: 28 of 73 females have been diagnosed as pregnant, which represents a percentage of 38%.

At birth, one of the calves obtained presented a different sex than the predicted one (female instead of male) which leads to an accuracy of 96.4% of the polymerase chain reaction sexing method (figure 3).

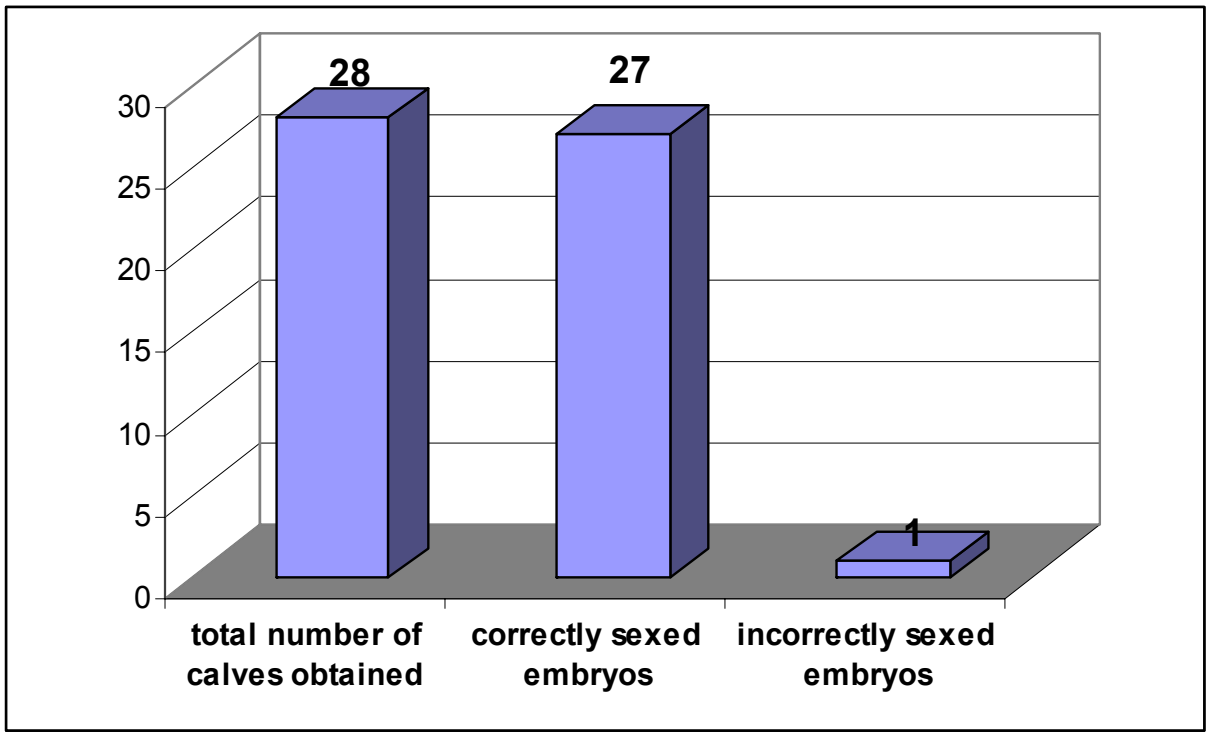

Figure 3. Accuracy of the PCR method of bovine embryo sexing

After performing the experiences in the blastomeres belonging to ***batch 2***, the following results have been obtained:

From the 76 embryos obtained, 72 (94.7%) had reached the morula stage, while 5 (5.3%) were in the early blastocyst stage. In what the quality of embryos was concerned, 74 embryos (97.4%) were transferable and were used for embryo biopsy while 2 (2.6%) could not be used for this purpose.

The fluorescent in situ hybridization and the amplification of the fluorescent signal using the tyramide kit yielded the following results:

- **41 samples** presented the characteristic fluorescence and thus we considered the blastomeres to come from male embryos

- **33 samples** did not present any fluorescence and thus we considered the blastomeres to come from female embryos.

When evaluating the pregnancy rate in recipient cows in which sexed embryos had been transferred, the following results have been obtained: 30 of 74 females have been diagnosed pregnant, which represents a percentage of 40.54%.

At birth, 4 of the calves obtained presented a different sex from the predicted one (females instead of males) which leads to an accuracy of 86.66% for the sexing of bovine embryos using the fluorescence in situ hybridization (figure 4).

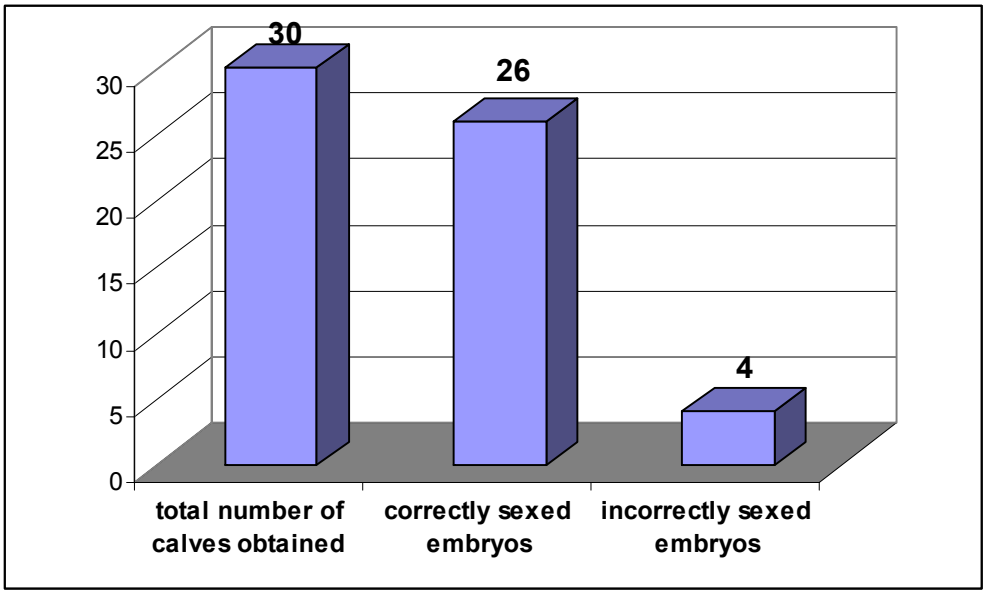

Figure 4. Accuracy of the FISH bovine embryo sexing method

Comparing the results obtained for the two methods of bovine embryo sexing, several observations can be made. The pregnancy rates obtained after transferring the biopsied embryos is almost similar for the two batches (around 40%), which is explainable as the biopsy technique did not differ between the two sexing methods. Other authors reported slightly higher pregnancy rates. Machaty et al. [12] transferred nineteen sexed morulae into recipient females on the seventh day after fertilization, and 25 days later ten of the recipients (52.6%) were found to be pregnant by ultrasonography. Shea [15] obtained a 58% pregnancy rate after transferring non-frozen sexed embryos, while Lopes *et al.* [11] found no statistical significant difference in the pregnancy rate between the bisected (60%) and fresh intact embryos (61%). Improvements of the biopsy technique have to be made in the future in order to reduce the damage of the embryos and to be able to obtain higher pregnancy rates.

In what the accuracy of the two methods is concerned, the PCR sexing yielded better results than the FISH method, being at the same time easier to perform and requesting lower expenses for materials and equipments. In other studies, the coincident rate of sex determination between biopsied blastomeres and matched demi-embryos by FISH was 96.0% (Lee *et al.* [10]) while the efficiency of sex determination by PCR was 90% when no control primers were employed and 94% using the co-amplification of SlS2/ClC2 primers pair; the accuracy of sexing, relating to the correct sex diagnosis of the calves, was 79% and 100%, respectively, for this two PCR-strategies (Lopes *et al.* [11]). Another paper reported an accuracy of sex prediction of 100% when the blastomere(s) dissociated from a morula exceeds more than three (Zoheir and Ahmed [17]).

Recent developments in the field showed that hundreds of thousands of offsprings have been born as a result of AI with sexed sperm. The technology for sexing sperm using flow cytometry has not changed greatly in the past 7 years, but refinements have speeded up the process and reduced damage to sperm. The process of commercialization of sexed sperm has accelerated recently. However, this technology is characterized by high costs, complexity of implementation and lower pregnancy rates than with control sperm. Nevertheless, sexed, frozen bovine sperm are being produced commercially in many countries, although from a limited number of bulls. As costs decline, sexed sperm will be used increasingly for cattle breeding (Seidel [14]).

Conclusions

The current study showed that the pregnancy rates obtained after transferring the biopsied embryos were of 38% in batch 1 and 40.54% in batch 2, being almost similar as the biopsy technique did not differ between the two sexing methods. The accuracy of the PCR method of bovine embryo sexing was of 96.4%, one of the embryos having a different sex than the predicted one. The accuracy of the FISH method of bovine embryo sexing was of 86.66%, four of the embryos having a different sex than the predicted one. The PCR sexing of bovine embryos yielded better results than the FISH method, being at the same time easier to perform and requesting lower expenses for materials and equipments. Improvements of the biopsy technique have to be made in the future in order to reduce the damage of the embryos and to be able to obtain higher pregnancy rates. We recommend the commercial use of PCR sexing kits for bovine embryo sexing, the FISH method being appropriate only for research purposes.

Acknowledgements

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