Preliminary report on sexing bovine pre-implantation embryos under the conditions of Portugal

Relatório preliminar da sexagem de embriões pré-implantação de bovino em Portugal

L. Lopes da Costa¹, J. Chagas e Silva², P. Diniz², R. Cidadão²

¹Faculdade de Medicina Veterinária, núcleo de Reprodução – CIISA, Rua Prof. Cid dos Santos, Polo Universitário, Alto da Ajuda, 1300-477 Lisboa, Portugal
²Divisão de Selecção e Reprodução Animal, Rua Elias Garcia 30, Venda Nova, 2704-507 Amadora, Portugal

Summary: Preliminary results of sexing pre-implantation bovine embryos by PCR amplification of chromosome-Y specific DNA fragments of biopsy samples from embryos in vitro produced and from embryos recovered from superovulated dairy donors of commercial herds are reported for the first time in Portugal. In Experiment 1, 50 good quality, morula and blastocyst stage embryos originated from in vitro matured, fertilized and cultured oocytes were used, from which 48 (96 %) had a sex diagnosis. In Experiment 2, 86 good (n = 73) and poor (n = 13) quality embryos recovered from 13 superovulated dairy donors were submitted to the sexing procedures. From these, 78 (90 %) had a sex diagnosis, although in 8 (9 %) of them an unambiguous diagnosis could not be given. The embryos were recovered at the herd and transported to the laboratory. After the biopsy sample was obtained the embryos were frozen or sent back to the herds, at which time the sexing results were given by phone. The pregnancy rate in 33 recipient heifers for good (n = 26) and poor (n = 7) quality embryos was 50 % and 14 %, respectively. Considering the sex of the calves born, the accuracy of sexing was 93 % (13 out 14). In conclusion, the efficiency and accuracy of the sexing procedures are compatible with the commercial use of sexing in embryo transfer programs in dairy cattle.

Resumo: Neste artigo são apresentados os resultados preliminares do primeiro programa português de sexagem de embriões pré-implantação de bovino, após amplificação por PCR de fragmentos de ADN específicos do cromossoma Y, obtidos de biópsias de embriões produzidos in vitro e de embriões recolhidos de dadoras superovuladas. No Ensaio 1, 50 embriões de boa qualidade, no estádio de morula ou blastocisto, obtidos de oócitos maturados, fertilizados e cultivados in vitro foram submetidos a sexagem, 48 (96 %) dos quais tiveram diagnóstico do sexo. No ensaio 2, 86 embriões de boa qualidade, de mães (n = 73) e de mães (n = 13) qualidade, recolhidos de 13 dadoras superovuladas foram submetidos a sexagem. Destes 86 embriões, 78 (90 %) tiveram diagnóstico do sexo, mas em 8 (9 %) desses, um diagnóstico inequívoco não pôde ser indicado. Os embriões foram recolhidos na vacaria e transportados ao laboratório. Após a obtenção da biópsia, os embriões foram congelados ou transportados para a respectiva vacaria, sendo o resultado da sexagem comunicado por telefone. A taxa de gestação de embriões de boa qualidade (n = 26) e de mães (n = 7) qualidade submetidas a biópsia e transferidos a fresco para 33 novilhas receberam foi de 50 % e de 14 %, respectivamente. Considerando o sexo dos vitelos nascidos, a precisão da sexagem foi de 93 % (13 em 14). Em conclusão, a eficiência e a precisão do método de sexagem são compatíveis com a sua utilização comercial.

Introduction

In Portugal, the actual dairy cattle industry places a much higher value in purebred female calves (for replacement or sale) than in male calves (virtually with no commercial value). Male calves born following the use of expensive reproductive technologies represent a major economic loss for the herd. The use of sexed embryos can increase the economic efficiency of embryo transfer programs (Willett and Hillers, 1994) and the rate of genetic gain both at the herd and at the selection nucleus level (Colleau, 1991). Pre-implantation bovine embryo sexing was achieved by cytogenetic (Picard et al., 1985), immunological (Booman et al., 1989) and metabolic methods (Tiffin et al., 1991) or by using male-specific chromosomal DNA probes (Bondioli et al., 1989). However, only the use of chromosome-Y specific DNA probes, together with the use of the polymerase chain reaction (PCR) for the amplification of embryo-derived biopsy samples, present efficiency and running speed compatible with a non-research use (Herr and Reed, 1991; Bredbacka et al., 1995).

PCR amplification of DNA fragments allows for the use of a small size biopsy sample, which is of great value for early cleavage stage in vitro produced embryos (Macháty et al., 1993), for splitted embryos (Bredbacka et al., 1994), for parent donor embryos for cloning by nuclear transfer (Le Bourhis et al., 1998) or for multiple genotype analysis (such as sex, marker genes or abnormal genetic mutations) (Chrenek et al., 2001). The major drawbacks of embryo biopsy are the associated increased risk of contamination by pathogens (IETS, 1998) and the decreased viability after freezing (Gustafsson et al., 1994; Thibier and Nibart, 1995; Shea, 1999).

After developing a commercially available sexing protocol in our laboratory we are now on a preliminary basis evaluating the efficiency of the sexing procedures.
and the pregnancy rate of both in vitro and in vivo produced biopsied pre-implantation bovine embryos.

Materials and methods

In Experiment 1, the efficiency of the sexing procedures was evaluated in 50 in vitro produced bovine embryos. The in vitro embryo production system was that previously described (Marques et al., 1995) with minor modifications. In this system, oocytes aspirated from ovaries collected at slaughterhouse are in vitro matured and in vitro fertilized with in vitro capacitated sperm and the obtained zygotes are in vitro cultured in a granulosa cell monolayer co-culture system for 7 to 8 days. Only good quality (grades 1 and 2; IETS, 1998), compact morula or blastocyst stage embryos were used. In Experiment 2, the efficiency of the sexing procedures and the pregnancy rate of the sexed embryos were evaluated with in vivo produced bovine embryos (n = 86). Donor cows (n = 13) were superovulated, inseminated and the ova recovered 7 to 8 days later and evaluated for stage and quality, using methods previously described (Lopes da Costa et al., 2001). At the herd, morula and blastocyst stage, embryos of good (n = 73) and poor (n = 13) quality (grades 1 to 3; IETS, 1998) were loaded in 0.25 ml French straws and then transported at environmental temperature to the laboratory. After the biopsy was obtained the embryos were evaluated for quality and loaded in 0.25 ml French straws and were either frozen or transported back to the farm, at which time the results of sexing were given by phone. Upon this information, the farmer decided which embryos (number and sex) were to be transferred. In order to make a preliminary evaluation of the pregnancy rate of fresh biopsied sexed embryos, transfers were done to previously selected recipient heifers (n = 33), according to methods described elsewhere (Lopes da Costa et al., 2001). Good (n = 26) and poor (n = 7) quality embryos were transferred.

The sexing procedures were those previously described by Herr and Reed (1991), and AB Technology (1993). Briefly, the embryos were washed in serum and BSA free medium and placed, one at each time, in a 50 µl medium micro-drop inside a 90 mm diameter Petri dish, under an inverted microscope (CK2, Olympus, Japan). A biopsy sample was obtained from each embryo using a microblade tool attached to a hydraulic micromanipulator (MO 155, Narishige, Japan). A biopsy sample was obtained from each embryo using a microblade tool attached to a hydraulic micromanipulator (MO 155, Narishige, Japan). The biopsy sample size was about 5 % to 10 % of the embryo size (Figure 1a). For blastocyst stage embryos the biopsy was taken from the trophoectoderm cells (Figure 1b). The biopsy sample was then transferred into a capillary PCR tube containing cresol solution, after which the DNA Y-specific probe (YCD Reagent, AB Technology, Inc.) was added. After running the PCR program (1 hour) the contents of the capillary tubes were dispensed into the wells of a 3 % agarose gel. After electrophoresis (25 minutes), the gel was transferred to an UV transilluminator for reading. In each sexing session, three controls were run (no DNA content, male and female controls). The presence of a non-specific DNA band and of a primer band indicated that a viable biopsy sample and an active DNA probe were present in the reaction tube, respectively. A specific band identified the male biopsy samples, while the female biopsy samples were identified by the absence of this band (Figure 2).

Figure 1 - a) Morula stage, good quality embryo recovered from a superovulated cow, after the biopsy procedure. The biopsy sample is about 10 % (6 to 8 blastomeres) of the main embryonic mass. The zona pelucida is sectioned and two fragments of it are shown on each side of the biopsy sample. b) Blastocyst stage, good quality embryo obtained by in vitro procedures. The microblade is cutting a biopsy sample from the trophoectoderm cells.

Figure 2 - Photograph of the agarose gel after electrophoresis of one sexing session of embryos from a superovulated donor. In positions 1 and 2 the no DNA control (only the primer bands are present); in position 3 the male control (from up to down, the inespecific autosomal DNA band, the Y-chromosome specific band and the primer band); in position 6 the female control (up, the inespecific autosomal DNA band and down, the primer band); in positions 4,5,8,9 and 13, male diagnosis; in positions 7,10 and 11, female diagnosis; in position 12, a result of no diagnosis (absence of DNA bands).
The efficiency of the sexing procedures was measured by the percentage of embryos with a sex diagnosis and by the accuracy of sexing for embryos that developed to term. Pregnancy diagnosis of recipients was done at 60 days of gestation by ultrasound and the sex of the calves recorded. Data were analysed by standard descriptive statistical methods and Chi square tests.

**Results**

In both experiments no DNA contamination was detected as judged by the controls run in each session. The efficiency of the sexing procedures is shown in Table 1. In Experiment 1, 48 out 50 (96 %) *in vitro* derived embryos had a sex diagnosis. In Experiment 2, 78 out 86 embryos (90.1 %) had a sex diagnosis. However, in 8 of these 78 embryos, the biopsy samples developed faint male-specific bands, therefore only 78 out 86 (81 %) biopsies originated an unambiguous sex diagnosis. The sex of these embryos was subjectively assigned as probable male (n = 5) or probable female (n = 3), depending on the presence or almost absence of the band, respectively. The biopsy samples of the remaining 8 embryos did not develop bands, so a sex diagnosis could not be given. The percentage of embryos with no sex diagnosis was higher, although not significantly, for poor quality embryos than for good quality embryos (23.1 % versus 6.8 %, respectively).

The DNA of these dead or degenerated cells could be viability, the biopsy sample consisted mainly of degenerated embryos, in order to do not further compromise their viability, the biopsy sample consisted mainly of degenerated cells excluded from the main embryonic mass. The DNA of these dead or degenerated cells could be already denaturated, which could affect the PCR amplification.

Absence of non-specific, male-specific and primer bands is observed in cases where the amplification of reaction products by PCR does not occur or in cases of fail to dispense the biopsy sample into the PCR reaction tube. This latter is a particularly sensitive step of the sexing procedure when using capillar PCR tubes and was a probable cause for the absence of a sex diagnosis of 4 % and 9 % of the *in vitro* and of the *in vivo* produced embryos, respectively. In poor quality embryos, in order to do not further compromise their viability, the biopsy sample consisted mainly of degenerated cells excluded from the main embryonic mass. The DNA of these dead or degenerated cells could be already denaturated, which could affect the PCR amplification.

Embryos recovered from superovulated donors, the efficiency obtained in this experiment was 81 %. This is lower than that reported in other studies (Le Bourhis et al., 1998: 90 %; Thibier and Nibart, 1995: 95 %). However, in a large six-year retrospective study reported by Shea et al. (1999), the between-year variation of the percentage of biopsy samples with undetermined sex ranged from 6 % to 18 %. Only a few studies (Thibier and Nibart, 1995) report the percentage of biopsies that give ambiguous sex diagnosis 2 %. If these are included, the efficiency of sexing of this experiment raises to 90 %.

An ambiguous sex diagnosis was obtained from 9 % of the *in vivo* embryos. Faint male-specific bands might be produced by an incomplete PCR amplification reaction, by a too small size biopsy sample or by a degenerated biopsy sample. Alternatively, non-specific bands might be produced due to DNA contamination or to irregular migration after electrophoresis. This latter was probably the case of the embryo diagnosed as probable female that originated a male calf, as no DNA contamination was detected in all sexing sessions. Thibier and Nibart (1995) reported that whenever the sex diagnosis is not straight forward, no attempt should be made to assign a sex to the embryo.

Table 1 Efficiency of sexing dairy cattle embryos.

<table>
<thead>
<tr>
<th>Embryo Quality</th>
<th>n</th>
<th>Unambiguous</th>
<th>Ambiguous*</th>
<th>No diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>In vitro</em> Good</td>
<td>73</td>
<td>61 (83.6 %)</td>
<td>7 (9.6 %)</td>
<td>5 (6.8 %)</td>
</tr>
<tr>
<td><em>In vitro</em> Poor</td>
<td>13</td>
<td>9 (69.2 %)</td>
<td>1 (7.7 %)</td>
<td>3 (23.1 %)</td>
</tr>
<tr>
<td><em>In vivo</em> All</td>
<td>86</td>
<td>70 (81.0 %)</td>
<td>8 (9.0 %)</td>
<td>8 (9.0 %)</td>
</tr>
</tbody>
</table>

* Embryo assigned as probable male or probable female

Table 2 Accuracy of sexing of dairy cattle embryos.

<table>
<thead>
<tr>
<th>Embryo sexing diagnosis</th>
<th>n</th>
<th>Calvings</th>
<th>Correct Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>22</td>
<td>10</td>
<td>10 (100 %)</td>
</tr>
<tr>
<td>Male</td>
<td>4</td>
<td>1</td>
<td>1 (100 %)</td>
</tr>
<tr>
<td>Probable male</td>
<td>3</td>
<td>1</td>
<td>1 (100 %)</td>
</tr>
<tr>
<td>Probable female</td>
<td>4</td>
<td>1</td>
<td>1 (100 %)</td>
</tr>
<tr>
<td>No diagnosis</td>
<td>1</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>14</td>
<td>13 (93 %)</td>
</tr>
</tbody>
</table>

The pregnancy rate in recipient heifers was 50 % and 14 % for good and poor quality embryos, respectively. Considering the sex of the calves born, the accuracy of sexing was 93 % (13 out 14; Table 2). One embryo assigned as probable female originated a male calf.

**Discussion**

The efficiency of the sexing procedures in providing an unambiguous sex diagnosis here reported for *in vitro* produced embryos (96 %) is similar to that described by Macháty et al. (1993): 95 %. For embryos recovered from superovulated donors, the efficiency obtained in this experiment was 81 %. This is lower than that reported in other studies (Le Bourhis et al., 1998: 90 %; Thibier and Nibart, 1995: 95 %). However, in a large six-year retrospective study reported by Shea et al. (1999), the between-year variation of the percentage of biopsy samples with undetermined sex ranged from 6 % to 18 %. Only a few studies (Thibier and Nibart, 1995) report the percentage of biopsies that give ambiguous sex diagnosis 2 %. If these are included, the efficiency of sexing of this experiment raises to 90 %.

One problem faced by the team was that farmers wanted all embryos to be sexed, irrespective of their quality. The results showed that poor quality embryos gave a higher percentage of no sex diagnosis and a lower pregnancy rate after transfer, compared to good quality embryos. Also, it is expected that poor quality embryos are not suitable for freezing. Therefore, the number of offspring of the desired sex from donors that yield a low percentage of good quality embryos can be
quite low. This might give a negative perspective of the technique to the farmers.

Distance between the herd and the lab is also of practical importance. For herds far from the lab (say over 2 to 3 hours travel), fresh transfers after returning to the herd (about 8 to 10 hours after embryo recovery) might negatively affect embryo survival. However, freezing also negatively affects in vivo survival of biopsied sexed embryos (Gustafsson et al., 1994; Thibier and Nibart, 1995; Shea, 1999). These aspects are currently being evaluated under the conditions of Portugal. Another possibility is to perform the biopsies at the farm level, freeze the embryos, hard-freeze the biopsies and send them to lab for sex diagnosis (Thibier and Nibart, 1995).

In conclusion, the efficiency and accuracy of the sexing procedures are compatible with the commercial use of sexing in embryo transfer programs in dairy cattle.

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References


