# A bovine protocol for training professionals in preimplantation genetic diagnosis using polymerase chain reaction

*Carlos Gilberto Almodin, M.D., Ph.D.,*[a,b] *Antonio Fernandes Moron, M.D., Ph.D.,*[b] *Luiz Kulay, Jr., M.D., Ph.D.,*[b] *Vânia Cibele Minguetti-Câmara, M.Sc.,*[a] *Andréa Cristina Moraes, Ph.D.,*[b] *and Maria Regina Torloni, M.D., M.Sc.*[b]

[a] Materbaby—Reprodução Humana e Genética, Maringá, Paraná, Brazil; and [b] Obstetrics Department, Universidade Federal de São Paulo, São Paulo, Brazil

**Objective:** To develop a bovine protocol for training in preimplantation genetic diagnosis (PGD) using PCR.
**Design:** Randomized study.
**Setting:** Human reproduction PCR laboratory.
**Patient(s):** Cow ovaries obtained from slaughterhouses.
**Intervention(s):** The ovaries were punctured and the oocytes were matured and submitted to in vitro fertilization. On the third day after fertilization, the embryos were biopsied and 1–2 blastomeres removed. A blastomere and the rest of the embryo were submitted to PCR for sex determination.
**Main Outcome Measure(s):** Establishment of a possible training protocol.
**Result(s):** A total of 50 embryos and 50 biopsied blastomeres were submitted to DNA amplification for sexing. Of the 50 embryos, 41 (82%) achieved successful DNA amplification and 9 (18%) did not. Of the 50 biopsies, 31 (62%) amplified and 19 (38%) did not. In 27 (65.9%) of the 41 embryos with DNA amplification, sex was identified as female and in 14 (34.1%) as male. In 40 cases (80%) amplification and sex determination were successful in both embryos and blastomeres. Sex was identical in all these cases.
**Conclusion(s):** This training model seems to be useful in identifying mistakes and difficulties and improving the professional's performance in the various stages of preimplantation genetic diagnosis. (Fertil Steril® 2005;84: 895–9. ©2005 by American Society for Reproductive Medicine.)

**Key Words:** PGD, training, bovine, embryos, model

In the last two decades, owing to the advances in the field of assisted reproduction, our knowledge of reproductive physiology has greatly progressed. It is now possible not only to actively participate in the fertilization process but also to manipulate embryos before they are transferred to the uterus.

With the advent of the new biopsy techniques, it is now possible to safely extract 1 or 2 blastomeres from 3-day-old embryos or remove trophectoderm cells from embryos in blastocyst stage (1). These small fragments can then be submitted to fluorescence in situ hybridization (FISH) or polymerase chain reaction (PCR) to investigate at the molecular level the characteristics of the early human embryo, such as chromosomal or genetic anomalies.

Preimplantation genetic diagnosis (PGD) is an emergent technology, which was implemented slightly more than ten years ago, when the first embryo biopsies were developed (2, 3). Handyside et al. (4) and Verlinsky et al. (5) were the first investigators to test PGD for X-linked and recessive autosomic diseases in humans.

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Reprint requests: Carlos Gilberto Almodin, Av. XV de Novembro, 1232, Maringá, Paraná, Brazil, CEP 87.013-230 (FAX: 55 (44) 225 1162; E-mail: almodin@materbaby.com.br).

However, PGD requires high-level techniques in taking samples and high-level knowledge about genetic diseases. In diagnosis, 100% efficiency and accuracy should be reached, but samples for PGD are so small that there is always a risk of failure (6). Thus, the first and main problem is how to train in, practice, and master all the technical details of the procedure without breaking ethical principles. Allowing untrained personnel to manipulate human embryos in order to acquire expertise would undoubtedly be considered unethical and possibly immoral.

Teams who intend to incorporate PGD in their work should therefore be encouraged to adopt a training protocol that allows individuals to manipulate animal embryos using the same equipment involved in human procedures. There are many animal protocols described in the literature, and preferences vary according to the objectives of the study. At this time, there is no standardization of the qualification of the personnel involved in PGD (7).

This study reports on a protocol developed based on a bovine model for training PGD using PCR, which seems to be useful in identifying mistakes and difficulties and improving the professional's performance.

## MATERIALS AND METHODS

The original protocol was established according to international norms of animal protection and was sent to the Ethics Committee of São Paulo Federal University. After its final approval in May 2001, cow ovaries were obtained from a local slaughterhouse, without any selection criteria, and transported in Dulbecco's phosphate-buffered saline (D-PBS) (Gibco Laboratories, Grand Island, NY) to the laboratory facilities at Materbaby—Reprodução Humana e Genética, where the experiment was carried out until its conclusion in May 2003.

### Fertilization and in Vitro Culture

In vitro fertilization was carried out using a modified Pavlock technique (8). Follicles 2–5 mm in diameter were aspirated and the follicular fluid examined using a stereoscopic microscope to identify the cumulus-oocytes. These were washed in D-PBS and incubated in 500 μL Medium 199 (Earle's salt) (Sigma Chemical Co., St. Louis, MO) supplemented with estrous cow serum (20%), FSH (10 μg/mL) (Serono, São Paulo, Brazil), calcium lactate (5.5 mmol/L), sodium pyruvate (2.3 mmol/L), sodium bicarbonate (9.5 mmol/L), Hepes 5.9 mmol/L), L-glutamine (0.7 mmol/L), streptomycin (15.6 mg m/L), and penicillin (7.5 mg/mL) (Sigma Chemical Co.) in Nunc four-well multidishes (Nuclon, Copenhagen, Denmark). Maturation was carried out at 38.5°C in an atmosphere containing 5% $CO_2$ in air with maximum humidity. After the maturation period (24–26 h) the oocytes were fertilized with swim-up–selected frozen-thawed bull spermatozoa according to Parrish and Susko-Parrish (9). Approximately $1 \times 10^6$ spermatozoa/mL were added to the dishes containing the oocytes, which had been transferred to the fertilization medium prepared according to Bavister and Yanagimack (10). After incubation of the gametes for 18–24 h, the probable pre-embryos without cumulus cells were deposited on Vero cells in the same maturation medium, without FSH, and with bovine fetal serum (Nutricell, Campinas, São Paulo, Brazil) instead of estrous cow serum.

### Embryo Biopsy

Five days after fertilization, the embryos were analyzed to determine their stage of development. Only embryos with eight or more cells were selected for blastomere aspiration and then were rinsed several times in D-PBS. The procedure was carried out as described by Verlinsky and Kuliev (11), using MMO-204D micromanipulators and IM6 injectors (Narishige Co., Tokyo, Japan) attached to a Diaphot Nikon inverted microscope (Nikon, Tokyo, Japan) with Hoffman phase contrast.

Biopsy was carried out in microdrops of HTF medium with Hepes (Conception Technologies, San Diego, CA) and bovine fetal serum overlaid with equilibrated mineral oil (Sigma Chemical Co., Charlottesville, VA). Under the microscope, the embryos were anchored by a holding pipette (Humagen, VA) with gentle suction. The embryo was positioned so that the blastomere to be biopsied was located at 12 o'clock. With the embryo secured in place, a microneedle (Humagen) was passed through the zona pellucida at 1–2-o'clock position and tangentially through the perivitelline space and out at the 10–11-o'clock position.

The embryo was then released from the holding pipette and held by the microneedle, which was brought to the end of the holding pipette and pressed against it, pinching a portion of the zona pellucida. By gently rubbing the microneedle against the holding pipette in a sawing motion, a cut was carried out and the embryo released.

Afterwards, the oocyte was rotated so that the opening was at the 12 o'clock position, and the microneedle was again introduced through the zona pellucida perpendicular to the first opening, so that after friction against the zona pellucida the two openings resembled an X. The opening was then positioned at 3 o'clock, and the aspirating micropipette (10-MBB; Humagem) was carefully placed at the orifice and one to two blastomeres were removed by gentle suction. One of these blastomeres was selected for PGD.

### Polymerase Chain Reaction

The selected blastomere and the remaining embryo (which was kept as control) were washed several times in D-PBS, transferred to 0.2 mL PCR tubes containing 5 μL sterile nuclease-free distilled water (Midwest Scientific, St. Louis, MO) and frozen to release DNA from the cells. The material was then thawed at room temperature, and 2 μL proteinase K (20 mg/mL) (Gibco) was added to digest the cellular cytoplasm in order to facilitate access to the DNA. The tubes were incubated in a thermal cycler (PTC 100; MJ Research, Waltham, MA) at 56°C for 15 min to activate the proteinase K and then reset at 95°C for 15 min for inactivation.

After this procedure, 20 μL of the reaction mixture (1 U Taq DNA polymerase, nucleotides [1.25 mmol each], 1.5 mmol $MgCl_2$, 15 mmol Tris-HCl pH 8.0, and 20 pmol of the pair of oligonucleotides [Gibco]) was added to the material to be amplified.

For amplification, two pairs of primers were used, one external and one internal ("nested" PCR), prepared according to Aasen and Medrano (12). The sequences of the external primers were (5′ primer) 5′-ATAATCACATG GAGAGGCACAAGCT-3′ and (3′ primer) 5′-GCACTT CTTTGGTATCTGAGAAAGT-3′ (Gibco). The length of the amplification product is 447 base pairs (bp). The internal primers used were (5′ primer) 5′-CAGAAGACAAAT GTCA-3′ and (3′ primer) 5′-TGGGAAGCATTTCTCCAT GCTGGGG-3′ (Gibco). The amplification of this region produces fragments of 397 bp. Thirty-three points of mutation have already been detected in 397 bp, excluding the 50 bp corresponding to the external primers. Some of these mutation points include sites that are digested by restriction enzymes.

The amplification was carried out in a thermal cycler (Perkin Elmer Cetua, Norwalk, CT). The first step was the

**TABLE 1**

**Results of PCR in embryos and blastomere biopsies.**

| | Embryo | | | | | |
|---|---|---|---|---|---|---|
| | Amplified | | Nonamplified | | Total | |
| Blastomere biopsy | n | % | n | % | n | % |
| Amplified | 31 | 62.0 | 0 | 0.0 | 31 | 62.0 |
| Nonamplified | 10 | 20.0 | 9 | 18.0 | 19 | 38.0 |
| Total | 41 | 82.0 | 9 | 18.0 | 50 | 100.0 |

*Note:* McNemar test $P=.002$

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complete DNA denaturation (95°C for 45 min). The second step consisted of 35 cycles of amplification at the following temperatures: 95°C for 15 seconds for DNA denaturation, 65°C for 1 min for annealing of the oligonucleotides, 72°C for 1 min for polymerization of DNA chains, and finally primer extension at 72°C for 5 min.

The tubes containing the PCR product were once again incubated in the thermal cycler at 94°C for 5 min for complete DNA denaturation and then submitted to 50 cycles of amplification: 93°C for 30 seconds for denaturation, 50°C for 15 seconds for primer annealing of the oligonucleotides, and 72°C for 1 min for polymerization of DNA chains (nested PCR).

The product of the nested PCR was submitted to the action of the Pst I and FOK I restriction enzymes (Gibco), which "splices" regions specific to sex determination. For each 10 μL of the final PCR nested product, 10–20 μL of each restriction enzymes was added, and the solution was kept in a double boiler at 37°C for 3 h. The product was electrophoresed on a 2% agarose gel (Gibco) and observed using a transilluminator equipped with ultraviolet light and a filter for 254 nm or 320 nm. If only one band of the bovine-specific product was visible on the gel the blastomere was considered to derive from a female embryo, whereas the presence of two bands indicated a male embryo.

In parallel, negative control reactions were carried out containing all reagents except DNA, which was replaced by the same volume of sterile water. The positive controls with male and female bovine DNA were prepared in the same manner as described above, substituting the solution containing the micromanipulated material with the same volume of control DNA.

The McNemar test was used for statistic evaluation of the results of embryos and biopsies submitted to PCR. Differences were considered significant if $P<.05$.

## RESULTS

A total of 50 embryos and 50 blastomeres from these embryos were submitted to DNA amplification for sex determination. Of the 50 embryos, 41 (82%) completed successful DNA amplification and 9 (18%) did not. Of the 50 biopsy samples, 31 (62%) had amplification and 19 (38%) failed to amplify. Cases that successfully completed DNA amplification in both samples (embryo and biopsy) and yielded identical sex identification were considered concordant. Cases in which just one of the samples amplified, and therefore did not permit comparison of results, were designated discordant. In all samples that amplified, sex was identical in both embryos and biopsies. As seen in Table 1, in 10 cases (20%) the results were discordant and in 40 (80%) they were concordant ($P<.002$). Of 41 embryos with amplifications, 27 (65.9%) were female and 14 (34.1%) were male. Figures 1 and 2 show some of the nested PCR results after Pst I and FOK I enzyme digestion. Figure 1 shows the negative agarose gel to confirm the nonamplification of the embryo 2 and biopsy 2A.

## DISCUSSION

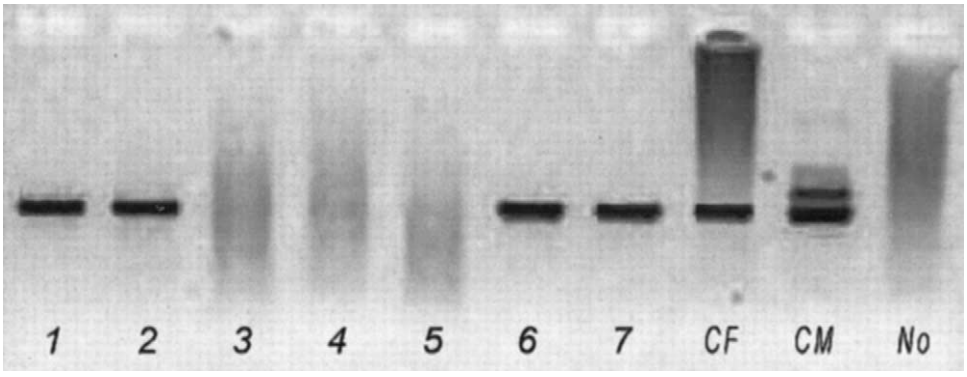
Polymerase chain reaction is probably the most manageable and powerful technique in the PGD field at present (13). It can be carried out by using various techniques, such as digestion restriction, heteroduplex analysis (14), and conformational polymorphism with a single strand (15), among others. All of these protocols start with amplified DNA extracted from the biopsied cell.

Sexing using PCR involves the coamplification of the Y-chromosomal sequence and an autosomal sequence which acts as control for presence of biopsy material and appropriated conditions for amplification. PCR is designed to yield different fragment sizes for the Y-chromosomal and the autosomal product, which are separated by electrophoresis using ethidium bromide–stained agarose gel. Success in sexing using PCR varies considerably owing to the different PCR protocols as well as the differences in carrying out the biopsy and DNA (13).

The first reports on amplification of single sequences were not very efficient and produced confusing results owing to Taq polymerase, which tended to incorporate more and more mistakes as cycles were repeated, especially when starting

## FIGURE 1

PCR product after Pst I enzyme digestion. The results confirm that the amplified fragments originate from bovine X chromosomes. Slot 1: embryo 1 (female); slot 2: biopsy 1A (female); slot 3: embryo 2 (no amplification); slot 4: biopsy 2A (no amplification); slot 5: control without DNA; slot 6: embryo 3 (female); slot 7: biopsy 3A (female); slot 8: CF (control female, fragment of 344 bp); slot 9: CM (control male, one fragment of 344 bp and another of 103 bp); slot 10: no (control without DNA).

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with only one cell and 6 pg of DNA. Primers of the nested PCR are those internal to the first pair of primers. The largest fragment produced in the first PCR cycle is used as a model for the second cycle. The use of nested PCR greatly improved the sensitivity and specificity of DNA amplification (12), and this is the reason we elected this technique in our study.

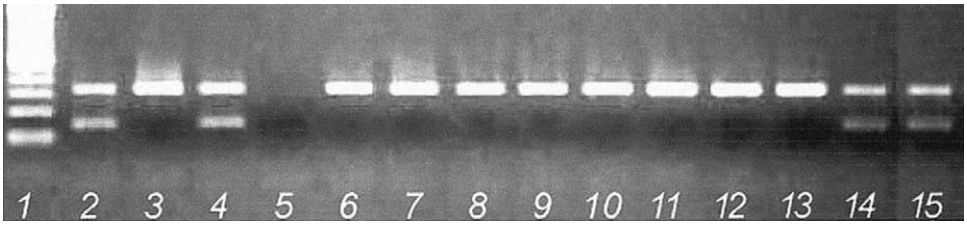
Using the training protocol suggested, we managed to amplify 82% of the embryos and 62% of the blastomeres, making sex determination possible. The difference between amplification rates of blastomeres and embryos was statistically significant and may have been due to difficulties in handling, with the probable loss of the biopsied material before amplification.

Other parameters may also have been involved in this difference, such as the nonextraction of DNA or DNA degradation in the test tube before the assay was carried out, because all methods for DNA liberation may cause damage to it. It is also believed that besides technical difficulties related to the procedure anuclear cells also may have been biopsied, which would lead to nonamplification. However, care was taken so that the selected biopsied blastomeres were incompatible with fragmentation material, which makes us believe that this problem could not have significantly interfered with the results.

In commercial settings, bovine PCR sexing efficiency (proportion of diagnosable samples) of 90%–95% has been reported (16–19). When the PGD was carried out in the embryo it was expected that amplification rates were close to those reported in the literature. However, we obtained an efficiency rate of 82%, which was probably because a greater

## FIGURE 2

Nested PCR for sex determination in embryos/biopsies. Digested with Pst I. Slot 1: marker; slot 2:control male; slot 3: control female; slot 4: embryo 12 (male); slot 5: biopsy 12A (no amplification); slot 6: embryo 13 (female); slot 7: biopsy 13A (female); slot 8: embryo 14 (female); slot 9: biopsy 14A (female); slot 10: embryo 15 (female); slot 11: biopsy 15A (female); slot 12: embryo 16 (female); slot 13: biopsy 16A (female); slot 14: embryo 17 (male); slot 15: biopsy 17A (male).

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amount of cytoplasm was present in the PGD assay, interfering with the results.

In our experiment, standard IVF insemination was used to generate and make available for training a fair amount of embryos in a short space of time. Apart from being less costly, it is ethical and still the most widely practiced form of insemination. This procedure, however, runs the risk of problems with the DNA in spermatozoa that may be bound to the zona pellucida and could contaminate the assay. To minimize the contamination problem, the blastomeres were removed by aspiration, and both embryos and blastomeres were rinsed several times. In 62% of the cases of this study, when both embryos and biopsies completed successful amplification sex determination was identical in 100% of cases (Table 1). This observation may seem to indicate that there was no contamination with foreign DNA.

The main objective of this training protocol is to provide conditions in which the experiment may be carried out as many times as necessary until safe rates are achieved in the procedure. Nevertheless, when a PGD diagnostic must actually be carried out in a human embryo, we believe that intracytoplasmic sperm injection should be the insemination procedure of choice.

Arguably, performing and handling the biopsy is the most laborious and critical component in PGD. Aspiration and microsection are the main approaches to biopsy for bovine sexing. Aspiration may be technically too demanding for widespread implementation, which has made microblade biopsy (microsection) the preferred choice in bovine embryos (20). The advantage of aspiration is that a very small sample can be taken with minimal damage to the embryo and zona pellucida, and that is why it is the most widely used technique in biopsies with human embryos.

In our experience we noticed that, in fact, the aspiration of bovine embryos obtained in vitro is more difficult to achieve than in human embryos. Blastomeres of bovine embryos seem to have a more fragile cellular membrane that can break easily, besides being darker and more compacted. This extra difficulty make us believe that when practitioners have achieved dexterity in manipulating bovine embryos, they would be ready to manipulate human embryos with safety and obtain good results.

In conclusion, we believe that by using this protocol, the professional would be ready to carry out PGD using PCR in human embryos when differences in amplification rates in both biopsied blastomeres and embryos are not significant. The outcome of this experiment clearly highlights the technical difficulties in safely and accurately handling embryos and the necessity for a carefully designed training program.

We believe that the protocol suggested here holds the potential to fill the gap in the qualification of PGD professionals. Besides using easily and cheaply obtained bovine ovaries, it is ethical and offers the opportunity for training in conditions very close to reality. Nevertheless, effectiveness of the protocol could be demonstrated only with the actual implementation of a training program by a representative number of PGD laboratories.

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