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

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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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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 1×10^6 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