A COMPARISON BETWEEN HEAT AND COLD SHOCK TREATMENTS IN AMPLIFICATION OF ZFX/ZFY GENE FOR EMBRYO SEXING

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SUMMARY

Early *in vitro* fertilised (IVF)-derived blastocysts were bisected using a stainless steel micro-blade attached to a micro-manipulator. A small portion of the bisected samples was then exposed to either heat or ultra-cold shock treatment to burst the blastomere cell membrane. The treated samples were then subjected to polymerase chain reaction (PCR) amplification of the ZFX/ZFY gene using P1-5EZ and P2-3EZ primers. The sex of each sample was diagnosed based on the presence of *Pst* I-RFLP on the PCR amplified DNA of the ZFX/ZFY locus. Both heat and ultra-cold shock treatments gave similar amplification of the ZFX/ZFY gene.

Keywords: *In vitro* fertilised (IVF)-derived embryos, bisection, sex determination, polymerase chain reaction (PCR)

INTRODUCTION

There is a great commercial importance in controlling the sex ratio of domestic animal species in agriculture. The ability to pre-select the sex of offsprings of agriculturally important livestock has long been an objective of animal breeders. There have been several approaches in the past to accomplish this task (Van *et al.,* 1989). Some approaches, such as the use of X-linked enzymes and serological assay involving H-Y antigen (William, 1986), are known as the non-invasive method. These methods, however, are not always accurate. Techniques like cytogenetic analysis (Betteridge, 1989) and the use of Y-specific DNA probes (Leonard *et al.,* 1987), which is one of the most accurate approaches are more invasive and therefore, limit the accessibility of embryonic material for biopsy. The Y-specific probe hybridization analysis, such as southern blotting and dot blotting still lacks sufficient sensitivity, and requires more than a week to perform. A far greater accuracy and improved sensitivity is provided by the polymerase chain reaction (PCR), which amplify the Y-related chromosomal gene for detection. The PCR method, undoubtedly, is a breakthrough procedure for embryo sex determination in ruminants (Peura *et al.,* 1991).

Sex determination of embryos has become commercially feasible when it is coupled with the artificial insemination and embryo transfer technologies. The use of ZFX/ZFY gene to determine the sex of cattle embryos was first introduced by Aasen and Medrano (1990). Although the gene was reported to be conserved in both X-chromosome (ZFX) and Y-chromosome (ZFY) (Schneider *et al.,* 1989), the ZFY gene was shown to have a unique *Pst* I restriction site, which is not present in the ZFX homolog (Aasen and Medrano, 1990). This feature enable one to distinguish ZFY from ZFX and thus, to use it to indicate the presence of the Y-chromosome for sex determination.

The objective of this study was to compare two different methods of cell disruption for the ZFX/ZFY amplification from bisected embryo cells. This was carried out as an attempt to develop a simplified procedure for determining the sex of bovine pre-implanted embryos prior to the transfer to recipient females. Both normal and hot start PCR amplifications of ZFX and ZFY genes were utilized while the accuracy of the technique was determined using the DNA from animals of known sex.

MATERIAL AND METHODS

Oocyte collection

Fresh ovaries were collected from a local abattoir, and were brought to the laboratory within 4 h of collection in normal saline at 25-30°C. Oocytes were collected from follicles of size ranging from 2-6 mm by aspiration with 18G needle. The oocytes were then placed into Modified Dulbecco's Phosphate Buffered Saline (DPBS). The cumulus-oocyte complexes (COC), which were completely surrounded by cumulus cells were selected and used in the *in vitro* maturation.

Culture of oocytes

Following selection, the oocytes were washed twice in DPBS (Gibco BRL, USA) and once in maturation medium, which comprise of tissue culture medium (TCM) 199 containing Earle's salt and 25 mM Hepes (Gibco BRL, USA) and supplemented with 5%

(v/v) foetal calf serum (Gibco BRL, USA). The oocytes were then introduced into a 100 μL maturation medium droplet, which was overlayed with mineral oil (SIGMA, USA). The oocytes were cultured for 19-22 h in a CO_2 incubator (5% CO_2 in air) at 39°C.

Sperm preparations

The stored frozen semen was thawed and washed two times by centrifugation at 450 g for 5 min in BO medium (Brackett and Oliphant, 1975), which contained 0.02mg/mL heparin. The spermatozoa concentration was re-adjusted to 6 x 10^6 sperm/mL using the BO medium, which contained 10 mg/mL bovine serum albumin (BSA). Calcium-ionophore (Cat. No. A 23187; SIGMA, USA) at a concentration of 0.1 μM was added to the sperm solution and hold up to 1 min. Sperm micro-drops of 100 μL, covered with mineral oil was then prepared.

Insemination and in vitro culture

After incubation for 19-22 h in a CO_2 incubator (5% CO_2 in air) at 39°C, the cumulus-oocyte complexes (COC) were washed in BO medium (Brackett and Oliphant, 1975) and then inseminated by transferring them into the sperm microdrops at the rate of 10-15 oocytes/drop. Following 6 h insemination, the cumulus cells surrounding the embryos were removed by pipetting. The embryos were then transferred into 50 μL CR1aa medium micro-drop and cultured *in vitro,* in a CO_2 incubator at 39°C for 7 days to allow further embryo developments (Fig. 1). Blastocysts that developed after the 7 days of *in vitro* culture were used for the experiment.
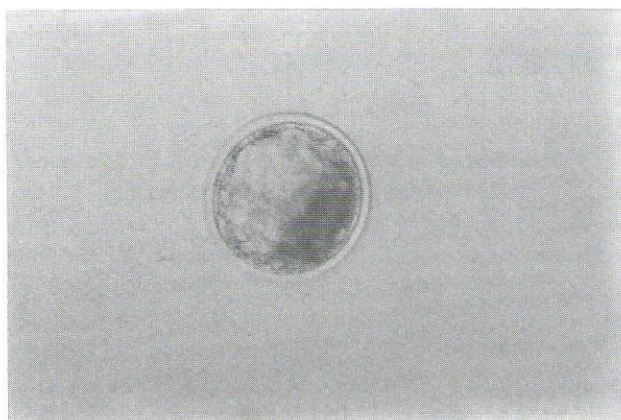

Fig. 1. The embryo was placed on a petri dish for *in vitro* culture

Embryo bisection

Prior to the bisection, selected blastocysts were washed 4 times in PBS (without BSA) and were then bisected using a sterile stainless steel micro-blade (Feather Bio-Cut Blade, Japan) attached to a micro-manipulator (Narashige, Japan). About one fourth of the blastocyst in the trophoblastic vesicle portion was bisected (Figs. 2 and 3). The bisected cells were then washed 3 times with PBS and placed in a sterile 500 μL microfuge tube. New sterile blade was used in each bisection in order to avoid contamination.
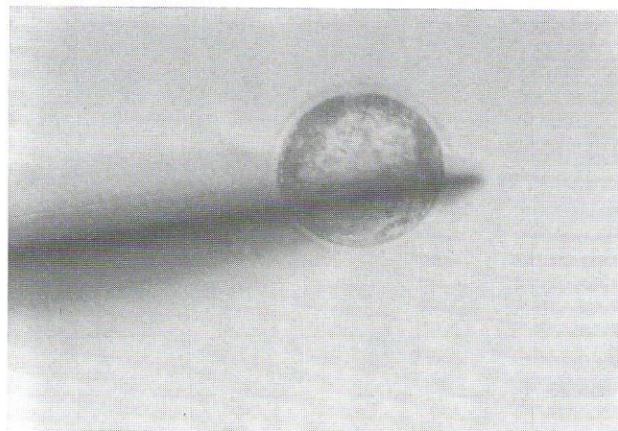

Fig. 2. The microblade is pressed down slightly until flattening of the embryo can be observed
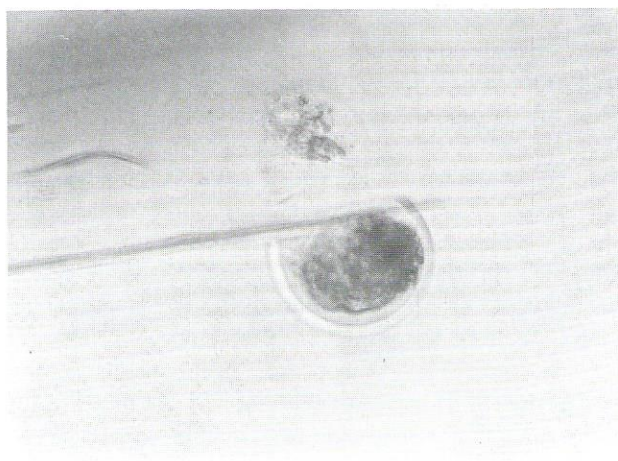

Fig. 3. The microblade is moved to one side of embryo and is pressed against the bottom of the dish to cut of the biopsy

Disruption of cells from embryo biopsies

After the bisected cells had been washed 3 times, 90 μL of the PCR mixture was added. The PCR mixture consisted of 10 μL of magnesium chloride ($MgCl_2$)-free 10x buffer, 10 pmole of each the primers, 5 IU of *Taq* DNA polymerase, 200 μM of dNTPs, and 2.5 mM $MgCl_2$. Two different treatments were used to distrupt the cells; the heat and ultra-cold treatments. The heat-shock treatment involved the exposure of the bisected cells in the PCR mixture

to 100°C for 5 min. A drop of mineral oil was added into the mixture to prevent condensation. The ultra-cold-shock treatment involved plunging of the bisected cells, which was in the PCR mixture into liquid nitrogen for three times. Samples were then left to return to the room temperature following each treatment.

A total of six IVF-derived cattle's embryos were used in this experiment. Three bisected embryos were treated with heat-shock while the remaining were treated the cool-shock.

Polymerase chain reaction

The PCR amplification was carried out using the MJ Research Thermalcycler (Winsconsin, USA) according to the parameters described by Shaharum *et al.*, (1995) as follows: denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds and extension at 73°C for 1 min. The PCR was carried out for 33 cycles. The hot start PCR was also carried out using the same parameters, but with preheating at 97°C for 5 mins in the first cycle. At the same time, a negative control was prepared by replacing embryo samples with the medium used to wash the embryos.

Restriction analysis and gel electrophoresis of PCR products

Fifteen µL of the PCR mixture was digested with *Pst* I (Promega, USA) at 37°C for 8 hrs.

Five µL of loading buffer, containing glycerol, bromophenol blue and xylene cyanol, was added into 10 µL of each sample before loading into the gel. Electrophoresis was carried out in 2% agarose gel in 1x TBE buffer. Following electrophoresis at 100 volts for 90 mins, the gel was stained with ethidium bromide (0.5 µg/mL) in the dark for 10 mins. The gel was viewed and then photographed over UV light.

RESULTS

A positive amplification of 440 bp of PCR product was achieved only after a slight modification, by an additional of 3 cycles and a single hot start trial. Initial amplifications using ordinary PCR procedure failed. The entire procedure of sex determination by ZFX/ZFY gene amplification could be completed within 6 to 8 h.

Amplifications of the ZFX/ZFY genes were successfully carried out following either heat-shock or ultra-cold-shock treatment. There was no difference in the amplification of the ZFX/ZFY genes between the heat-shock and the ultra-cold-shock treatments (Fig. 4).

The PCR products, which were identified as male embryos were cleaved by the *Pst* I into two parts of 100 bp and 300 bp, along with the 440 bp uncut portion of ZFX (Fig. 5). Of the six embryos used in this study, two were identified as male embryos while the remaining were identified as female embryos.
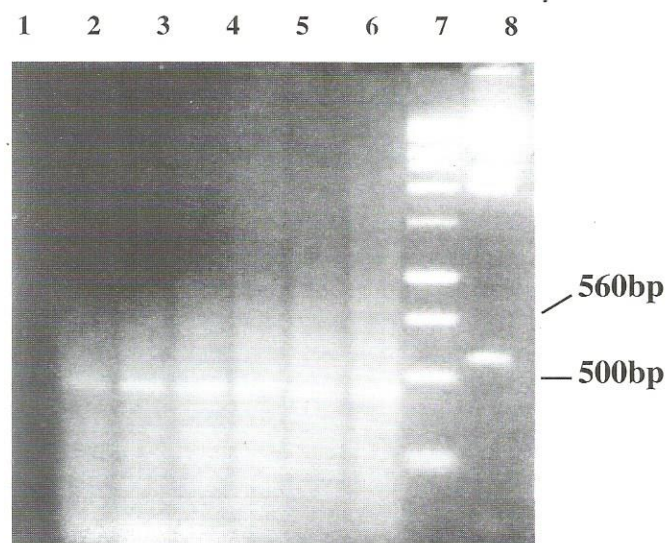

Fig. 4. Amplifications of the bisected embryos using the P1-5EZ and P2-3EZ primers showing the 440 bp sized PCR products (ZFX). Lanes 1, 2 and 3 are heat-shock treated and lanes 4, 5 and 6 are cold-shock treated
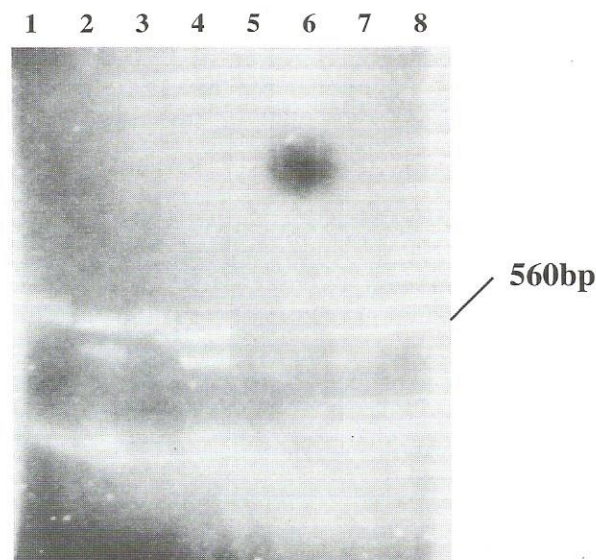

Fig. 5. Embryo sex determination using the ZFX/ZFY PCR assay. The restriction enzyme *Pst* I cuts the Y chromosomal homologue into fragments of 340 bp and 100 bp. The 440 bp long X chromosomal homologue remains intact. Lanes 1, 3, 7 and 8 are female embryos while lanes 2 and 4 are male embryos. Lane 5 is negative control and lane 6 is the Hind III marker.

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DISCUSSION

The sex of preimplanted embryos can be determined accurately using the PCR technique. Earlier studies using PCR have been carried out on bovine (Peura *et al.*, 1991; Agrawala *et al.*, 1992), mouse (Bradbury *et al.*, 1990; Kunieda *et al.*, 1992) and human (Handyside *et al.*, 1990) embryos. In those approaches, oligonucleotide primers were designated to the Y chromosome specific repetitive DNA sequences. This study, however, used the ZFX/ZFY genes as genetic markers for sex determination of preimplanted IVF derived bovine embryos.

The primary application of bovine sex determination assay is to determine the sex of bovine embryos. To ensure maximum embryo viability for the subsequent embryo transfer, sex determination assay should utilise a minimal amount of DNA, such as those obtained in a biopsy of 2-4 cells. Thus, successful sex determination requires the assay to be extremely sensitive. A few trials have actually been carried out using Proteinase K (Bredbacka *et al.*, 1995) as the membrane disruption agent. However, no amplification was detected due to the lack of template DNA of the cells that failed to burst. Following those failures, two different physical treatments were adopted to distrupt the cells; the heat shock and ultra cold shock.

This simple embryo bisection, coupled with PCR technique allow animal breeders to determine the sex of livestock preimplanted embryos before embryo transfer is conducted. However, it is better if less number of cells (2-3 cells) can be obtained through embryo manipulation to minimise the damage to the embryos.

ACKNOWLEDGEMENTS

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RINGKASAN

SUATU BANDINGAN DI ANTARA PERLAKUAN RENJATAN HABA DAN SEJUK DALAM PENINGKATAN GEN ZFX/ZFY UNTUK PENJANTINAAN EMBRIO

Blastosista terbitan IVF tersenyawa in vitro *telah dibelah mengguna mikropisau keluli yang tersambung kepada satu mikromanipulator. Sebahagian kecil daripada sampel terbelah itu kemudian didedahkan sama ada kepada perlakuan renjatan haba atau ultrasejuk untuk meletuskan blastomer membran sel. Sample terperlaku kemudian dikenakan peningkatan tindakbalas rangkaian polimerase (PCR) terhadap gen ZFX/ZFY dengan menggunakan primer P1-5EZ dan P2-3EZ. Jantina setiap sampel didiagnoskan berdasarkan kewujudan Pst I RFLP pada DNA tertingkat PCR pada lokus ZFX/ZFY. Kedua-dua perlakuan renjatan haba dan ultrasejuk menghasilkan kadar peningkatan sama terhadap gen ZFX/ZFY.*