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Single Cell Sexing Using a Part of Metal Razor Blade of Bovine Embryos Through Male Specific Repetitive DNA

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Abstract: The development of quick and sensitive methods for sexing of bovine embryos in early stage would be considerable in economic advantages. Polymerase Chain Reaction (PCR) were used for amplification of DNA sequenced derived from the male sex chromosome. The sexed method was allows to removed single cell biopsy using a part of metal razor blade from bovine embryos in less than two minutes without compromise developmental potential. The embryo biopsy can be used for male sex determination. Definite signals following PCR amplification were obtained in all cases indicated that single blastomere cell from preimplantation of bovine embryos was sufficient for male sex determination. Male sex determination of the embryos was developed further in both *in vitro* and *in vivo* successfully.

Key words: Single Cell · Bovine Biopsy · Sex Determinant

INTRODUCTION

Predeterminant of the sex embryos in farm animal's offspring could have a major impact on livestock production and would be considerable in economic advantages. To be usefully in embryo transfer industry, some sexing techniques should be efficient, accurate, rapid, simplification and without any detrimental effect [1]. The recent development of the Polymerase Chain Reaction (PCR) method was significant in this field because this technique allows amplification of male specific (Y-chromosome-specific) repetitive sequences and thus determination of the sex embryos in relatively short time and high reliability [2].

. Poernomo[3] have suggested that the cell number may be valid indicator of quality and viability of *in vitro* developed preimplantation embryos. Jiang *et al.* [4] was observed a variation of cell number in different grades of blastocyst derived from *in vitro* fertilization bovine oocytes. Blastocyst were developed slower where it both lower quality and fewer cell number than fast developed the counterparts. Geshi [5] was performed simultaneously through PCR amplification of DNA sequences derived from the chromosomes X and Y. The development of quick and sensitive methods for sexing embryos in early stages was important for cloning of bovine Y-specific sequences as well by the use of the PCR. The amplification of the autosomal satelite as an internal control for each sexing assay monitors the effective presence of bovine DNA in the samples and quality of the enzymatic reaction.

The purpose of the study was determined of the male sex embryos which had been collected from single blastomere cell biopsy through a fragment metal razor blades using male specific repetitive DNA.

MATERIALS AND METHODS

Single Cell Biopsy: Embryos were collected in five days following artificial insemination. Japanese Black cows as donor was flushed through Phosphate Buffer Solution (PBS) contained 10% Bovine Serum Albumen (BSA). Embryos collections were selected. Only 30 embryos which reached morula stage and good or excellent grade was used. Biopsied embryos were carried out in micro drops of PBS without BSA covered with mineral oil to avoid evaporation. In the Figure 1, biopsies were done by means a part of commercial metal razor blades glued on the Narishige micromanipulators which it attached on a Nikon inverted microscope, according to Poernomo previous method [3]. Zona pellucida was cut off and gently presses through zonae until blastomeres were

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glue

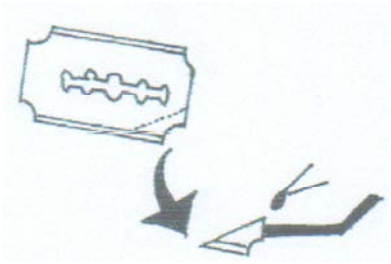

Fig. 1: Fragment metal razor blade glued onto glass micropipette

removed. Then cut single cell blastomeres and allows in the medium. All biopsied process need less than two minutes without compromised developmental potential. Biopsied embryos were transferred separately on micro drops PBS medium contained BSA and kept in the CO_2 incubator overnight. Biopsied embryos was reached blastula stage were transferred into the recipient.

Male Specific Repetitive DNA: Each single blastomere cell was washed five times in PBS contained 0.3% Poly Vinyl Alcohol (PVA), followed further once in pure water. Then transferred each single blastomere cell into 0.5 ml PCR tube contained 5 µl pure water. Tube contained blastomere cell were boiled one minute and then kept into the ice one minute more. The components for the amplification were added into the PCR tube to a final volume 10 µl. The reaction mixture namely PCR buffer (10 X) 1 µl, 2.5 mMdNTP's (final concentration 200 µM) 0.8 µl, 3.5 U/µl DNA polymerase (final concentration 0.5 – 1.0 U) 1.5 µl, and 2.9 µl water. Finally the reaction mixtures were overlaid with one drop (5-10 µl) mineral oil to avoid evaporation. Two pairs of primers were used for amplification both were 100 pmol/µl sense (forward) primer 0.075 µl and 100 pmol/µl antisense (reverse) primers 0.075 µl. The sequence of the primers as follows:

Male (Y) specific primers
Upstream 5'-CCCTTCCAGCTGCAGTGTCA-3'
Downstream 5'-GATCTGTAACTGCAACTGGC-3'

Bovine DNA specific primers
Upstream 5'-TGGAAGCAAAGAACCCCGCT-3'
Downstream 5'-TCGTGAGAAACCGCACACTG-3'

The length of the male (Y) chromosome specific amplification product was 190 base pairs (bp), instead length of the bovine DNA specific amplification product was 160 bp. The amplifications were carried out into DNA Thermal Cycler for 50 cycles each samples, consisted of template denaturation at 94°C for 60 seconds and primer annealing as follows;

97°C 5 seconds - 50°C 30 seconds - 72°C 20 seconds 5 cycles
97°C 5 seconds - 50°C 10 seconds - 72°C 10 seconds 45 cycles
Finally primer extension at 72°C for 5 minutes
After last cycle, samples were kept 4°C until electrophoresis

Agarose Gel stained through Ethidium Bromide were used for electrophoresis, followed further samples were evaluation by ultraviolet light. Results were done if only single band of the bovine specific product was visible on the gel the blastomeres cell was considered to derive from female embryo. On the contrary, whereas on the gel was presence of two bands it was referred to male embryo.

RESULTS

Biopsies were done less than two minutes each embryo without any detrimental effect to the whole embryos. Single blastomere cells from all 30 morula embryos were successfully biopsied (Figure 2). Only two blastomere cells were destroyed during embryo manipulation and washed. Remain 28 embryos were determined through PCR, whether male or female. Missing samples were easily detected by the absence of the bovine DNA specific signals.

Biopsies embryos kept in the CO_2 incubator overnight and then evaluation in the following morning. All biopsied morula embryos successfully reached early blastula stage. Because of total number of recipient cows, only ten embryos were transferred into ten recipient cows. Pregnancy evaluation after 25 days embryo transferred through transrectal ultrasound examination by a B-mode scanner (CS 9100®, Physia) through 5.0 MHz linear array transducer. Research result show five recipient cows were successfully pregnant, instead remain five cows were failed.

Sex was determined at all of 28 biopsied embryos because amplification of single blastomere cell could be allowed sex to be determined.

Evaluation through gel electrophoresis after PCR amplification was shown on the Figure 3. In lanes 3, 4 and 5, respectively, was visible two bands indicated presence of both male chromosome specific (190 bp) and bovine

Fig. 2: A pair of single blastomere cell

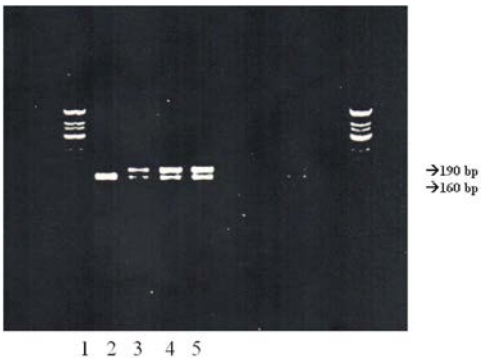

Fig. 3: In the lane 3, 4 and 5, respectively, was visible two bands indicated presence of both male chromosome specific (190 bp) and bovine specific fragments (160 bp)

specific fragments (160 bp). Presence of both chromosome referred sex of the embryo was male. On the lane 2 was visible only a single band. The length of the band was 160 bp referred bovine specific product, instead male chromosome specific product. Missing male chromosome specific product was referred embryo not male. However, the embryos had strong probability as female.

DISCUSSION

According to the previous research, some methods were held for embryos biopsied on the preimplantation embryo stage i.e. zona drilling using acidified solution, aspiration using a fine micro pipette, slice cut using a metal razor blade which it acrylic glued onto a glass tube [3]. Poernomo[6] was compared four biopsies methods on preimplantation embryo stage namely fine glass, fine metal, fragment glass and fragment metal. This study was shown only slight differences among them regarding their effect on the subsequent embryonic development.

On the previous study, a large number biopsied blastomere cells was used for bovine sex determination. Bredbacka *et al.* [7] was used equally half of whole morula stage to determined of sex bovine embryos by PCR method. Whether Ilmensee [8] was used bisected embryo on the blastocyst stage for sexed through DNA hybridization. These present research was demonstrated that DNA content on single blastomere cell of bovine embryos was sufficient for amplification and had possibility to sex determined through PCR method.

Owing the small size of the blastomere cells, it could be extremely useful, however, in the present study all samples contained embryonic material as revealed through these primers. Thus, the PCR method described above for sexing bovine embryos was very effective and it could be accomplished in more and less than 2 hours for a relatively larger of the embryos number. The single blastomere cell biopsies from each embryos should be caused very little trauma to the development of whole embryos. Biopsied embryo was not altered development potential energy *in vitro*. Following morning after incubation on the CO_2 incubator, all biopsied embryos were reached blastula stage. Blastula stage was a transferable stage because in this stage embryo was ideally to transfer *in vivo* into recipient. So, biopsied single blastomere cell embryos which it had no any detrimental effect achieved higher pregnancy rate than biopsied a large number of blastomere cells. The method described in this present study was suitable method for embryo sexing in *in vitro* and *in vivo* fertilization.

According to Ilmensee [9] was obtained a number of uncertain results if only male specific primers was used and one out of twelve has the Y specific signal was very weak. On the present study, we used two pairs of Y chromosome specific primers to avoid uncertain result and weaknesses signals.

Kadokawa *et al*. [10] reported that heterosexual chimeras among singleton females produced by multiple non-sexed embryo transfer were difficult to diagnose by chromosome typing, although it was detectable through PCR. This observation indicated that sexing method through PCR from a single blastomere cell of a preimplantation embryo provide correct results. Through bovine specific primers could be facilitated the detection of absence of the blastomere cell in the reaction mixture. Thus excludes the false female results. The negative control could rules out reagent contamination, but can't guarantee against sporadic contamination events. Fortunately, the odds sporadic contamination event occurred twice in the same way were very low [11].

Sex determination in single blastomere cells were followed further embryo transfer into recipient cows. Pregnancy evaluation after 25 days embryo transferred through transrectal ultrasound examination by a B-mode scanner through 5.0 MHz linear array transducer show five recipient cows were successfully pregnant, instead remain five were failed. The quick and sensitive biopsy procedure can be used as a routine production tools for rapid sex determination commercially.

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