

Sex Determination by PCR in Single Bovine Blastomeres Biopsied by Extrusion Method at the 8-cell Stage

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Abstract: We attempted to determine the sex by polymerase chain reaction (PCR) in single bovine blastomeres at the 8-cell stage. PCR was performed on male-specific primers attached to a bovine embryonic sex determination kit, an XY Selector. Embryos at the 8-cell stage were isolated by the EDTA method, and one (1/8 embryo), two (2/8 embryo), and four (4/8 embryo) blastomeres were subjected to PCR. The detection rates for male-specific PCR product were 25.8, 25.8 and 45.2% for 1/8, 2/8 and 4/8 embryos, respectively. In some embryos, despite detection of the male-specific product in 4/8 embryos, the male-specific product was not detected in 1/8, 2/8 or both 1/8 and 2/8 embryos derived from the same embryo (3.2, 3.2 and 16.1%, respectively). Collecting single blastomeres by the extrusion method affected neither the rate of development to blastocysts nor the number of cells in blastocysts. PCR was performed in 1/8 embryos collected by the extrusion method, and the male-specific PCR product was detected. Nevertheless, in 27.5% of embryos, despite detection of male-specific PCR product in 7/8 embryos, the male-specific product was not detected in the 1/8 embryo from the same embryo. These findings indicated that collecting single blastomeres at the 8-cell stage allows the selection of the male embryo by PCR, and also the extrusion method is useful for biopsy of embryo at the 8-cell stage.

Key words: Sexing, PCR, Bovine, Embryo, Biopsy

As sex determination methods for early mammalian embryos, a method using male-specific antigen, a method using sex difference in the rate of development,

a method which quantifies X chromosome-linked enzymes, a method for detecting the Y chromosome, and a method that amplifies Y chromosome-specific nucleotide sequences and analyzes the product have been investigated [1, 2]. Among these methods, the method using a male-specific antigen and the method using sex difference in the rate of development are not always accurate, and the overall sexing rates of the method for measuring enzymes linked to the X chromosome and the method for detecting the Y chromosome are low. Therefore, the method for detecting Y chromosome-specific nucleotide sequences by amplifying the sequence by polymerase chain reaction (PCR method) is considered to be promising, and has been developed for cattle [3–7], pigs [8] and mice [9, 10]. As an advantage of the PCR method, tests can be rapidly and accurately performed on only a few cells, and commercial use in cattle has begun, but with the current biopsy method, blastomeres are collected by excising mural trophectoderm cells with the zona pellucida at the blastocyst stage, and there is a risk of affecting the normal development of the fetus. In mice, the extrusion method has been established, in which blastomeres at the 4- or 8-cell stage are biopsied by extruding from a small slit made in the zona pellucida [11, 12]. Collecting samples for PCR by this method may reduce injury to the embryo.

Therefore, in this study, we collected single blastomeres at the 8-cell stage by the extrusion method, and attempted to determine embryonic sex by the PCR method and investigated the developmental potency of the remaining 7/8 embryos.

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Materials and Methods

1. Oocyte recovery, *in vitro* maturation and *in vitro* fertilization

Embryos at the 8-cell stage and blastocysts were produced by *in vitro* maturation and fertilization (IVM-IVF). Recovery of oocytes and IVM-IVF were performed following the method reported by Kameyama *et al.* [13]. Oocytes were cultured for maturation in TCM199 supplemented with 5% fetal calf serum (FCS) for 21 h. IVF was performed in m-BO medium [13] with frozen semen for artificial insemination. Spermatozoa were preincubated for 2.5 h for capacitation. After IVM, oocytes were incubated with capacitated spermatozoa at a density of $10\text{--}15 \times 10^6$ sperms/ml for 5 h. Presumptive zygotes were co-cultured with cumulus cells in TCM199 supplemented with 1% FCS. The medium was exchanged on days 3, 5 and 7 (day 0=IVF) after IVF. The 8-cell stage embryos on day 3 and the blastocysts on day 9 were used in the experiments.

2. Preparation of samples for PCR

Samples for PCR were prepared from one (1/8 embryos), two (2/8 embryos), four (4/8 embryos), seven (7/8 embryos) and eight (8/8 embryos) blastomeres, and blastocysts. The zona pellucida of 7/8 and 8/8 embryos and blastocysts was removed with PBS (–) containing 0.5% pronase. All embryos were washed 3–5 times with PBS (–) by means of siliconized pipettes, then transferred to 0.5 ml microtubes containing 8 μ l of sterilized pure water. The prepared samples were stored at -20°C until use for PCR.

The 1/8 embryos were prepared by the EDTA method and the extrusion method, 2/8 and 4/8 embryos by the EDTA method, and 7/8 embryos by the extrusion method. In the EDTA method, zona pellucida-removed embryos at the 8-cell stage were pipetted in PBS (–) supplemented with 0.02% EDTA, and each 1/8, 2/8 and 4/8 embryo was separated. In the extrusion method, 1/8 embryos was extruded with a micromanipulator, and the remaining blastomeres were used as 7/8 embryos. Micromanipulation was performed in M2 medium [14]. The outer and inner diameters of the holding pipettes were 180 and 25 μ m, respectively. Those of injection pipettes were 20 and 10 μ m, respectively, and the tip was beveled to 35° , and a spike was made by using a microforge. To extrude blastomeres, a part of the zona pellucida was incised, and M2 medium was blown into the embryo via the injection pipette that was inserted at another site.

3. PCR

PCR was performed following the instructions and using only male-specific primers attached to the bovine sex determination kit (XY Selector, Itoham Foods Inc.). An enzyme mixture solution was prepared at a ratio of 2 \times reaction solution: enzyme solution = 9.9 μ l: 0.1 μ l, and 9.5–9.9 μ l was added to a sample. As a PCR apparatus, QUICK THERMO PERSONAL (NIPPON GENETIC Co.) was used. The program consisted of 2 min initial at 95°C and 44 cycles of 30 sec at 95°C , 10 sec at 50°C , and 10 sec at 70°C . The PCR product was analyzed by mini-gel electrophoresis. To 15 μ l of the PCR product, 3 μ l of BPB solution (25% Glycerol, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol) was added, then loaded onto a gel. Electrophoresis was performed with 3% agarose gel (NuSieve GTG Agarose, FMC Bioproducts) at 50 V for approximately 70 min. After electrophoresis, the gel was stained with ethidium bromide for 50 min, and the appearance of the male-specific PCR product was examined under ultraviolet illumination.

4. Viability of 7/8 embryo biopsied by the extrusion method

Intact 8/8 embryos, 8/8 embryos kept on a warm plate (37°C) for a time required for micromanipulation (10 min), 7/8 embryos prepared by destroying one blastomere, and 7/8 embryos from which one blastomere was removed by the extrusion method were co-cultured with cumulus cells for 144 h (until day 9), and the development of embryos to blastocysts was observed. Destruction of a single blastomere was performed according to the extrusion method. The blastocysts derived from intact 8/8 embryos and 7/8 embryos prepared by removing one blastomere by the extrusion method were stained by double fluorescence staining [13, 15], and the numbers of cells in the inner cell mass and the trophectoderm were counted after 120 h of culture (day 8).

Results

1. Sex determination in 1/8, 2/8, and 4/8 embryos prepared by the EDTA method

The sex of the 1/8, 2/8, and 4/8 embryos prepared by the EDTA method was determined (Table 1). As the control, 8/8 embryos and blastocysts were used. The detection rates for male-specific PCR product were 25.8, 25.8, 45.2, 40.0 and 43.8% for 1/8, 2/8, 4/8, 8/8 embryos, and blastocysts, respectively. Sets of 1/8, 2/8, and 4/8 embryos derived from the same embryo were individually prepared, but the male-specific PCR product detection rates of each embryo in a set were not

consistent.

Consistency of sex determination among the 1/8, 2/8 and 4/8 embryos is summarized in Table 2. The ratio of embryo sets in which male-specific PCR product was detected in all embryos was 22.6%, and that of embryo sets in which no male-specific PCR product was detected was 54.8%, so that the percentage of embryo sets that obtained consistent sex determination was 77.4% (24/31). In the embryo sets with inconsistent determinations, male-specific PCR product was detected in 4/8 embryos, but not in 1/8 (3.2%) or 2/8 (3.2%), or both 1/8 and 2/8 embryo (16.1%).

2. Biopsy of embryos at the 8-cell stage by the extrusion method

The efficiency of collecting blastomeres by the extrusion method is shown in Table 3. Of 116 embryos tested, one or two blastomeres were collected in 78.4%

(a single blastomere extruded: 59.5%, two blastomeres extruded: 19.0%), but in 12.1% of the embryos, three or more blastomeres were extruded, and extruded blastomeres were destroyed in 9.5%.

In the 7/8 embryos obtained by removing one blastomere by the extrusion method, 60.9% developed to morulae, and 47.8% developed to blastocysts (Table 4). The ratio of development to blastocysts from the 7/8 embryos did not significantly differ from those from the intact embryos at the 8-cell stage (62.8%), from the embryos at the 8-cell stage kept on a warm plate for the time required for micromanipulation (50.0%), or from the 7/8 embryos prepared by destroying one blastomere (45.0%) ($P>0.05$).

The blastocysts derived from the 7/8 embryos prepared by the extrusion method consisted of 63.1 cells on average (Table 5). Among these cells, the number of cells in the inner cell mass (ICM) was 14.3 (22.7%).

Table 1. Sex determination by PCR in 1/8, 2/8 and 4/8 embryos produced by the EDTA method

Type of embryo	No. of embryos applied	No. and (%) of embryos detected male specific signals
1/8*	31	8 (25.8)
2/8*	31	8 (25.8)
4/8*	31	14 (45.2)
8/8	10	4 (40.0)
blastocyst	16	7 (43.8)

*Sets of 1/8, 2/8, and 4/8 embryos derived from the same embryo were individually prepared.

Table 2. Consistency of sex determined by PCR in sets of 1/8, 2/8 and 4/8 embryos produced by the EDTA method from the same embryo

Detection of male specific signals in			No. and (%) [*] of sets
1/8	2/8	4/8	
♂	♂	♂	7 (22.6)
♂	—	♂	1 (3.2)
—	♂	♂	1 (3.2)
—	—	♂	5 (16.1)
—	—	—	17 (54.8)

*Percentages were calculated by the total number of sets.

Table 3. Result of manipulation by the extrusion method at the 8-cell stage

No. of embryos manipulated	No. and (%) [*] of embryos classified by the result of manipulation as			
	Extrusion of one blastomere	Extrusion of two blastomeres	Extrusion over three blastomeres	Breakdown of blastomeres
116	69 (59.5)	22 (19.0)	14 (12.1)	11 (9.5)

*Percentages were calculated by the number of embryos manipulated.

Table 4. Effect of manipulation on the development of biopsied embryos

Treatment of embryo	No. of embryos cultured	No. and (%) of embryos developed to	
		Morula	Blastocyst
Control	43	32 (74.4)	27 (62.8)
Leaving for 10 minutes*	20	15 (75.0)	10 (50.0)
Destroy of one blastomere	20	14 (70.0)	9 (45.0)
Biopsy of one blastomere	23	14 (60.9)	11 (47.8)

*On warm plate (37°C).

Table 5. Numbers of cells in blastocysts derived from 7/8 embryos biopsied by the extrusion method

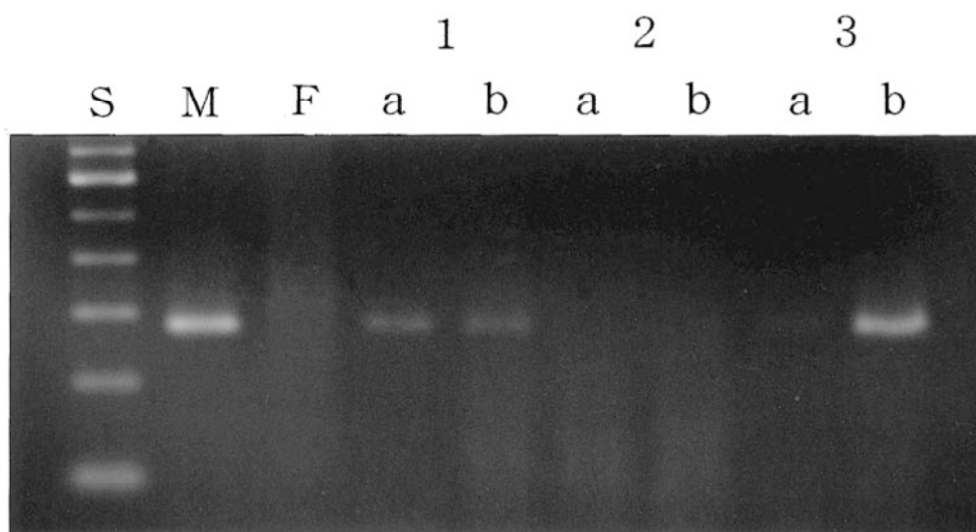
Origin of blastocysts	No. of blastocysts	Numbers of cells* and (%)** of		
		ICM	Trophectoderm	Total
7/8	10	14.3 ± 2.2 (22.7)	48.8 ± 6.8 (77.3)	63.1 ± 8.3 (100)
8/8	12	16.7 ± 2.5 (24.1)	52.4 ± 5.8 (75.9)	69.1 ± 7.3 (100)

*Mean ± S.E.

**Percentages were calculated by the total numbers of cells.

Table 6. Consistency of sex determined by PCR in 1/8 and 7/8 embryos produced by the extrusion method

Detection of male specific signals in 1/8 embryos	No. and (%) of sets classified by the detection of male specific signals in 7/8 embryos		
	♂	–	Total
♂	4 (10.0)	0 (0.0)	4 (10.0)
–	11 (27.5)	25 (62.5)	36 (90.0)
Total	15 (37.5)	25 (62.5)	40 (100.0)

**Fig. 1.** Agarose gel electrophoresis of PCR products from 1/8 (a) and 7/8 (b) embryos produced by the extrusion method. Each set was derived from the same embryo. S: size marker, M: purified male DNA, F: purified female DNA. Male-specific PCR products (about 300 bp) were detected in lane M, 1a, 1b and 3b.

These numbers of cells did not significantly differ from those of the blastocysts derived from the intact 8/8 embryos (total number of cells: 69.1, ICM cells: 16.7).

3. Sex determination in 1/8 and 7/8 embryos prepared by the extrusion method

The sex of 1/8 and 7/8 embryos prepared by the extrusion method was determined and the results were

classified by consistency (Table 6). The male-specific PCR product detection rates were 10.0% of the 1/8 embryos, and 37.5% of the 7/8 embryos, showing that the results were not consistent. The male-specific PCR product detection rate for the 1/8 embryos significantly differed from the theoretical value of 50% ($P < 0.05$). The embryo sets in which male-specific PCR product was detected in both 1/8 and 7/8 embryos was 10%, and

those in which male-specific PCR product was not detected in either 1/8 or 7/8 embryos was 62.5%. Therefore, consistency in sex determination was obtained in 72.5% (29/40) of the embryo sets. In the sets for which consistency was not obtained, male-specific PCR product was detected in the 7/8 embryos, but not in the 1/8 embryos (27.5%).

Discussion

In blastomeres separated by EDTA, we investigated the number of blastomeres required for sex determination of bovine embryos at the 8-cell stage. Kirkpatrick and Monson [5] collected 2–10 blastomeres from embryos at the stages from compacted morulae to expanding blastocysts, and determined the sex with male-specific and gender neutral primers. They reported that PCR product common to both sexes was detected in 95% of the samples, and the sex ratio (male ratio) was 65.8%. Macháty *et al.* [6] collected single blastomeres from embryos at the 16- and 32-cell stages, and determined the sex with male-specific and gender neutral primers. The detection rate for PCR product common to both sexes was 95%, but the sex ratio was not mentioned. In this study, only male-specific primers were used, so that samples in which male-specific PCR product was detected were regarded as male. In 4/8 embryos, 45.2% were male, which did not differ from the expected male ratio of 50%, showing that four blastomeres are sufficient to obtain almost completely reliable sex determination of bovine embryos at the 8-cell stage. The male-specific PCR product could be detected in single blastomeres at the 8-cell stage, showing that male embryos can be selected by using a 1/8 embryo.

In the PCR method, Y chromosome-specific nucleotide sequences are amplified and used for sex determination, the success rate in selecting sex for birth should be 100%. Nevertheless, because a success rate of less than 100% in cattle was mentioned in many reports [3, 5, 7], it is difficult to obtain 100% in cattle, unlike mice, for which 100% was reported [9]. In this study, determined sex was inconsistent among 1/8, 2/8, and 4/8 derived from the same embryo, and between 1/8 and 7/8 from the same embryo. Some male embryos may be regarded as female when determination is made with two or fewer blastomeres. As a reason why male-specific DNA was not amplified, sampling error, contamination during sampling (effects of FCS and BSA on enzyme reaction, contamination of DNA due to cell debris and spermatozoa adhering to the zona pellucida surface), and difference in heat conductance among

models of PCR apparatus were considered [16]. Because 1/8 and 2/8 embryos are small and easily adhere to the wall of a pipette, it is difficult to confidently put these embryos into PCR tubes. It is necessary to use siliconized pipettes and reduce the carry-in solution as much as possible.

On sex determination of the 1/8 embryos collected by the EDTA method or the extrusion method, male-specific PCR product was detected in both samples. In the extrusion method, a small amount of culture medium was blown into the zona pellucida to extrude blastomeres from a incision, and it is not necessary to strain blastomeres and nuclei by aspiration, unlike in the aspiration method or enucleation method. Therefore, blastomeres collected by the extrusion method are less injured at the cell membrane and nuclei, indicating that this method is appropriate for gene diagnosis including sex determination of embryos. But the male-specific PCR product detection rate in 1/8 embryos obtained by the extrusion method was lower than that in 1/8 embryos obtained by the EDTA method, suggesting that some blastomeres were injured during extrusion from the slit made in the zona pellucida. Injury to blastomeres can be prevented by making a larger slit in the zona pellucida, but the biopsied embryos for which hatching was made easier may be likely to be injured during embryo transfer. In this study, micromanipulation was performed in conventional M2 medium. Addition of sucrose and EDTA may cause constriction of blastomeres, and make separation easier, which may make the extrusion method easier and more reliable. Takeuchi *et al.* [17] reported that they collected human single blastomeres at the 4- and 8-cell stages by the enucleation, aspiration, or extrusion method, and the viability of embryos at the 4-cell stage was not affected by the aspiration or extrusion method, and the viability of embryos at the 8-cell stage was not affected by either method. In cattle, single blastomeres at the 16- to 32-cell stages collected by the aspiration method were transplanted, and fertility similar to that of the control subjects was obtained [6]. In this study, the 7/8 embryos obtained by the extrusion method developed to blastocysts without difference from the development of intact embryos at the 8-cell stage, showing that the collection of embryos by the extrusion method causes less inhibition of embryonic development.

The above findings showed that male embryos can be selected by the PCR method with a single blastomere at the 8-cell stage obtained by the extrusion method, and the extrusion method results in less injury to embryos. It is therefore useful for biopsy.

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