Sex Determination of *In Vitro* Fertilized Bovine Embryos by Fluorescence *In Situ* Hybridization Technique

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ABSTRACT

Sexing from bovine embryos which were fertilized *in vitro* implicate a possibility of the sex-controlled cattle production. This study was carried out to investigate the possibility of determining of embryo sex by fluorescence *in situ* hybridization (FISH) technique. FISH was achieved in *in vitro* fertilized bovine embryos using a bovine Y-specific DNA probe which constructed from the btDYZ-1 sequences. To evaluate Y-chromosome specificity of the FISH probe, metaphase spreads of whole embryos and lymphocytes were prepared and tested. A male-specific signal was detected on 100% of Y chromosome bearing metaphase specimens. Using the FISH technique with a bovine Y-specific probe, 232 whole embryos of 8 cell- to blastocyst-stage were analyzed. Observing the presence of the Y-probe signal on blastomeres, 102 embryos were predicted as male, and 130 embryos as female. The determining rate of embryo sex by FISH technique was about 93% regardless of embryonic stages. In conclusion, the FISH using a bovine Y-specific DNA probe is an accurate, reliable and quick method for determining the sex of bovine embryos.

(Key words : Fluorescence *in situ* hybridization, Sexing, *in vitro* fertilized embryo, Bovine)

INTRODUCTION

Embryo sexing from biopsied or splitted embryos which were fertilized *in vitro* or *in vivo* implicates a possibility of mass production of the sex controlled animal. To achieve a successful result, it requires many developed techniques, which involved in *in vitro* fertilization, micromanipulation, embryo freezing, embryo transfer, and gender analysis.

Various methods have been used for determining the sex of bovine embryos. The representative methods for determining the sex of mammalian embryos were the karyotyping at early stages (Wintenberger-Torres and Popescu, 1980; Picard et al., 1985; Sohn et al., 1996), using antibodies to search for male specific antigens (Wachtel et al., 1975; White et al., 1982; Anderson, 1987; Utsumi et al., 1991), detecting metabolic differences between male and female embryos (Williams, 1986) and using DNA hybridization to detect male-specific Y-chromosomal DNA sequences (Leonard et al., 1987; Bondioli et al., 1989).

Although numerous approaches for mammalian embryo sexing have been reported, the method of polymerase chain reaction (PCR) is well developed and prevailed (Herr & Reed, 1991; Bredbacka et al., 1995; Lopes et al., 2001; Park et al., 2001). This technique can amplify a male-specific DNA sequence within a few hours, providing accurate, reliable information for the sex of the embryos. Because of its convenience, the method of PCR provided the tool of bovine embryo sexing as the first commercial form of bovine preimplantation genetic diagnosis (Thibier and Nibart, 1995; Shea, 1999). In spite of the advantages of PCR, however, this method has some misjudgment from the amplification of non-specific sequences or contaminated association, and has impossible to detect the sex chromosomal mosaicism (Herr & Reed, 1991; Lee et al., 2003). Recently the fluorescence *in situ* hybridization (FISH) technique was introduced to another available method for embryo sexing (Kobayashi et al., 1998). FISH is to detect hybridization of nucleic acid probes with chromosomes using fluorescent tags. This method allows individual eukaryotic chromosomes to be colored fluorescently at the location of specific gene or DNA sequences. Because the hybridization signal of

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the probe and target gene was easily found under fluorescent microscopy, the FISH has become used for physical gene mapping and genetic diagnosis (Klinger et al., 1992.; Delhanty et al., 1993; Iannuzzi et al., 2003). FISH is also a rapid and simplified method that detects chromosomal aberration in human embryos produced by *in vitro* fertilization (Munne et al., 1993). Even though several bovine Y-specific sequences have been identified and their probes are available, there is little information for sexing a bovine embryo by FISH.

Therefore, this study was to validate the practice and accuracy of FISH method for sex determination of *in vitro* fertilized bovine embryos.

MATERIALS AND METHODS

Embryo production

Bovine embryos were produced by the *in vitro* maturation and fertilization and cultured for 7~8 days. Briefly, the ovaries of Holstein and Korean Cattle were obtained from a local slaughterhouse. Cumulus-oocyte complexes (COCs) were aspirated and then 30~50 oocytes of compact cumulus cells were transferred into TCM-199 medium (Invitrogen Life Technologies, San Diego, CA, USA). After culture for 22~24 h, COCs were washed and placed in 50 μ l BO medium (Brackett and Oliphant, 1975) supplemented with BSA and heparin. Using the frozen-thawed semen, the spermatozoa was washed twice with BO supplemented with caffeine. The 50 μ l of sperm suspension ($2\sim4\times10^6$ spermatozoa /ml) was introduced into the medium that contained COCs for fertilization. The oocytes were transferred into CR1 (Rosenkrans et al., 1993) on layers of bovine ovary ephithelial cells. Eight cell embryos were transferred into same medium supplemented with glucose at 72 h after post-insemination. Morulae were retransferred into same medium supplemented with glucose and FBS instead of BSA at 120 h after post-insemination.

Preparation of metaphase specimens

First, bovine metaphase chromosomes were prepared from *in vitro* blood culture. In brief, 0.5ml of whole blood were cultured in RPMI1640 supplemented with 1% penicillin-streptomycin, 1% pokeweed mitogen and 10% fetal bovine serum (v/v, all from Invitrogen Life Technologies, San Diego, CA, USA). The lymphocytes were incubated for 72 h at 37℃ in an atmosphere of 5% CO_2 and 95% air. Colcemid (0.1μg/mℓ, Invitrogen Life Technologies, San Diego, CA, USA) was added the culture for 50 min before the end of the incubation period. The harvested cells were incubated for 20 min with 0.06M KCl (Sigma Chem, St Louis, MO, USA) hypotonic solution. The swollen lymphocytes were fixed in a methanol/acetic acid (3:1) fixative solution. After repeating fixation procedure, the fixed cells were dropped onto glass slides and air-dried overnight.

Second, metaphase chromosomes from *in vitro* fertilized embryos of cattle were prepared as described previously (Sohn et al., 1996). Briefly, *in vitro* produced embryos were cultured in 0.5 mℓ CR1 medium containing 0.1 μ g/ ml colcemid solution for 6 to 8 h. After rinsing the embryos with PBS, the embryos were transferred to a drop of hypotonic solution (0.9% sodium citrate). Swollen embryos were treated with a series of fixation; a fixative solution (methanol and acetic acid, 3:1) was dropped into the hypotonic solution. An embryo placed on a slide glass was treated with a small drop of acetic acid dropped to separate the blastomeres, and then fixed with same fixative solution. If cytoplasm remained, it was removed by spreading one drop of another fixative, a mixture of methanol, acetic acid and water (4:3:1). The specimens were dried at room temperature for overnight.

Construction of bovine Y-specific DNA probe and FISH

To construct bovine Y chromosome-specific DNA probe for FISH, a partial btDYZ-1 sequences that had known as bovine male specific DNA were used (Perret et al., 1990). The w-5 (5'-GTGTGTGTGCTGTTCTCAAC-3') and w-3 (5'- CACACGACAAAATATTGCAC-3') PCR primer pair was designed to produce a 385-bp segment, which was a partial segment of btDYZ-1 sequences. A 385-bp DNA fragment was simultaneously labeled with digoxigenin (Dig)-dUTP (Boehringer-Mannheim, Indianapolis, IN, USA) and amplified by PCR using the w-5 and w-3 primer pair and male bovine genomic DNA as template following the manufacturer's instruction (Boehringer-Mannheim, Indianapolis, IN, USA).

The FISH procedure was slightly modified from that of Kobayashi et al. (1998). Briefly, the sample slides incubated in 2xSSC containing 5 μ g RNase A (Sigma, Saint Louis, MO, USA) for 30 min and then rinsed with ddH_2O. Treated slides were dehydrated with series of increasing ethanol. The hybridization buffer (40% 4xSSC, 50% formamide and 10% dextran sulfate) containing 100 ng Dig-labeled probe was applied onto the specimens. The specimens and probe were denatured for 10 min on a heating plate at 72℃ and were then allowed to hybridize for 1 h at 38.5℃. The slides were washed with 2xSSC at 72℃ for 5 min and subsequently with PN buffer (0.1% sodium phosphate, 0.1% Nonidet P-40) for 2 min at room temperature. The air-dried slides were incubated for 30 min at 38.5℃ with anti-Dig-fluorescein isothiocyanate (FITC) conjugate (Boehringer-Mannheim, Indianapolis, IN, USA). They were then washed with PN buffer, counter-stained with propidium iodide and examined under a fluorescence microscope (Model AX-70, Olympus, Tokyo, Japan) equipped with a WIB 523 nm-pass filter. The images were captured by digital CCD

(DP-70, Olympus, Tokyo, Japan). From each sample slide, all metaphases and interphase nuclei were evaluated for the Y chromosome-specific signal.

RESULTS

To confirm the fidelity of the bovine Y-chromosome specific probe on cytological specimens, we performed fluorescence *in situ* hybridization on the metaphase spreads and interphase nuclei of normal male and female bovine lymphocytes. Following FISH on the metaphase chromosome spreads using the 385-bp Dig-labeled probe, a hybridization signal on the Y chromosome was detected in male lymphocytes (Fig. 1a), but not in female cells (Fig. 1b). In uncultured lymphocytes, a fluorescein isothiocyanate (FITC) signal also exhibited on interphase nuclei of male lymphocytes, but not on female lymphocytes.

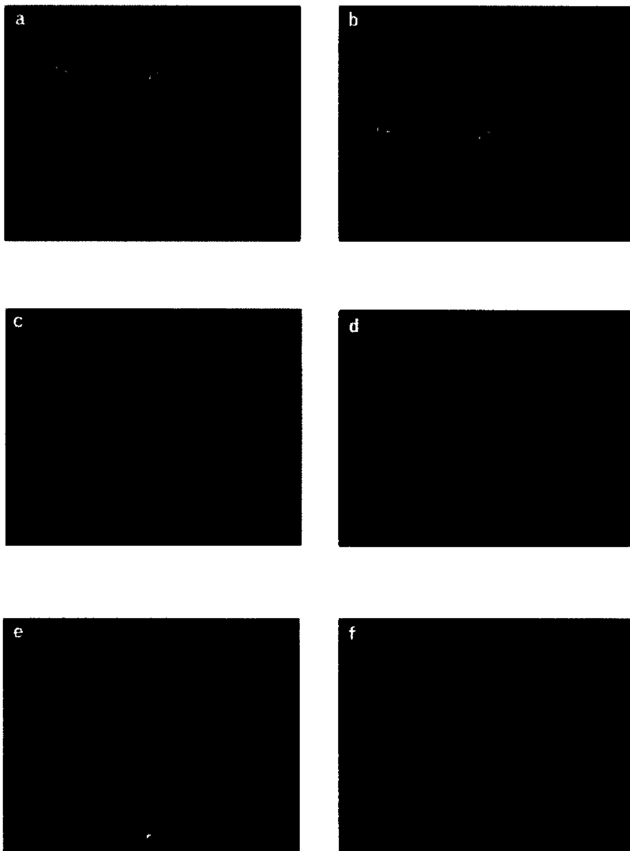

Fig. 1. Metaphase spreads and interphase nuclei with fluo scence *in situ* hybridization using a bovine Y-specific pro metaphase spread from male lymphocytes (a), metaphase spread from female lymphocytes (b), metaphase spread from bovine embryo consisting of XY chromosomes (c), metaphase spread from bovine embryo consisting of XX chromosomes (d), interphase nuclei from blastomeres, predicting as male (e), interphase nuclei from blastomeres, predicting as female (f).

According to this result, it was confirmed that our bovine Y-specific probe was specific to bovine Y chromosome. Therefore, we applied to FISH to bovine embryo specimens using the 385-bp Dig-labeled probe, a partial bovine btDYZ-1 sequences for embryo sex determination. To identify the embryo sex, cultured embryos were treated with colcemid before specimen preparation. Because of inhibiting spindle fiber by colcemid, these specimens showed metaphases and interphase nuclei of blastomeres. The XY chromosome bearing specimens had clearly exhibited a FITC signal on Y chromosome and interphase nuclei but no signal in XX bearing specimens (Fig. 1c. d). Some specimens had clear FITC signals on all of blastomeres but the others had no signal on any blastomeres. The embryo with the FITC signal was predicted as a male sex (Fig. 1e) and without signal was predicted as a female sex (Fig. 1f).

Table 1 showed the results of detection rate of the bovine Y-specific DNA signal on the blastomeres of bovine embryos. Total 250 embryos produced by *in vitro* fertilized protocol were examined. The developmental stages of examined embryos were 8-cells, 16-cells, molulae and blastocysts. Targeting the whole embryos, 232 embryos (92.8%) were available for FISH on embryonic sexing. According to the presence of FITC signal, 102 embryos (44.0%) had positive signals and 130 embryos (56.0%) had no signals. The embryos that had positive signals were predicted as the male sex. We could not observe any evidence for embryonic stage to affect the determination of sex. However, the embryos of 8-cell stage were less efficient than the other stages on the rate of analysis for FISH.

DISCUSSION

This study clearly showed that the FISH technique can

Table 1. Number of *in vitro* fertilized bovine embryos with FISH using a bovine Y-specific probe

Stage of embryos	Number (%) of embryos			
	Examined	Analyzable	With Dig-signal*	Without Dig-signal
8-cell	63	51 (80.9)	22 (43.1)	29 (56.9)
16-cell	18	17 (94.4)	8 (47.1)	9 (52.9)
Morula	70	68 (97.1)	31 (45.6)	37 (54.4)
Blastocyst	99	96 (97.0)	41 (42.7)	55 (57.3)
Total	250	232 (92.8)	102 (44.0)	130 (56.0)

* The Dig-signal implicates the presence of Y-chromosome.

accurately identify the sex of bovine embryos. This technique was also less time consuming, within 2 h. Using a partial bovine btDYZ-1 probe, the hybridization efficiency was 100% in male lymphocytes while the rate of the nonspecific hybridization was 0% in the female cells. These results also were confirmed with embryo specimens. Hybridization signals showed simultaneously on the metaphase spreads and on the interphase nuclei from the XY chromosome bearing embryo.

In bovine Y-chromosome specific DNA probes, many of bovine Y-specific sequences have been identified and their probes were developed. However, there is little information of the probes for sex determination of bovine embryos by FISH. The BC1.2 sequence was widely used as the FISH-probe in bovine embryo sexing (Cotinot et al., 1991; Kirszenbaum et al., 1990; Kobayashi et al., 1998). This sequence is a 54-bp motif which is present at about 2,000~2,500 copies in bovine male genome. It is also conserved within *Bos* and *Bison* genera and remains male specific. The BC1.2 probe was located on bovine Yp13 by *in situ* hybridization (Schwerin et al., 1992; Goldammer et al., 1997). Therefore, the male specificity and repeated nature of the BC1.2 sequence have enabled researcher to use it as a molecular probe for sex determination on small numbers of cells by *in situ* hybridization. In this study, we used a partial btDYZ-1 sequence as the bovine Y-specific probe for FISH. In the btDYZ-1 sequences, Perret et al. (1990) have cloned a bovine (Bos taurus) Y-specific sequence. This sequence is composed of 60 tandem repetitions of a motif consisting of two parts: a 40-bp-long unit separated from the next repeat by a TG-rich stretch varying in length between 12 and 63 bp. The number of copies of this repeated motif has been estimated at 6×10^4 per male genome. As a consequence, DYZ-1 might represent approximately 1/20 of the bovine Y chromosome. The btDYZ-1 has been mapped by *in situ* hybridization to the pericentric region of the Y chromosome. It has been also shown to be conserved within the *Bos* and *Bison* genera of the Bovidae subfamily (Perret et al., 1990). In our FISH results, a partial btDYZ-1 probe showed much more sensitive to hybridize with bovine Y chromosome than BC1.2 probe. We could not find any information for the FISH using the bovine btDYZ-1 probe.

The sex determination of bovine embryos was widely used on farms by PCR techniques (Thibier and Nibart, 1995; Shea, 1999; Lopes et al., 2001; Tominaga, 2004). Many reports showed that the PCR protocol used for embryo sexing ranged 92% to 95% of efficiency and 90 to 100% of accuracy (Kirkpatrick and Monson, 1993; Machaty et al., 1993; Thibier and Nibart, 1995; Shea, 1999; Lopes et al., 2001). Although the PCR is a reliable method for sex determination of bovine embryos, this assay system has some misjudgment from the amplification of non-specific sequences or contaminated association (Herr & Reed, 1991). In addition, the PCR cause a discrepancy in the sex when the embryos had mixoploid blastomeres or sex chromosomal mosaicism (Lee et al., 2003). In this respect, it is unlikely that FISH can be affected by such error factors in detecting the sex of bovine embryos.

In conclusion, FISH using a partial btDYZ-1 probe is very accurate and reliable method for the sexing of *in vitro* fertilized bovine embryos. Therefore, to achieve the mass production of the sex controlled animal, the FISH using a bovine Y-specific probe is likely to be a powerful tool for sex determination of bovine embryos.

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