Evaluation of Sexing in Boar Sperm Using Chromogenic *In Situ* Hybridization

H. H. Kim[1], D. E. Roh[1], T. K. Jo, J. W. Byun, J. W. Lee, Y. S. Kim, Y. J. Hwang and D. Y. Kim[†]

Division of Biological Science, Gachon University of Medicine and Science, Incheon 406-799, Korea

Chromogenic *In Situ* Hybridization을 이용한 돼지 정자의 성 선별

김효현[1] · 노다은[1] · 조태경 · 변진우 · 이정화 · 김윤섭 · 황유진 · 김대영[†]

가천의과학대학교 생명과학부

SUMMARY

돼지 정자의 성 선별에는 일반적으로 유속 세포 분석기를 이용한다. 유속 세포 분석은 DNA량의 차이에 기초하여 정자를 분리하는 기술로써 X 정자와 Y 정자를 90% 정확도로 분리할 수 있다. 그러나 이러한 유속 세포 분석 기술은 정자의 손상을 야기해 정자의 기능과 수정능에 영향을 미치므로, 본 연구에서는 특정한 핵산 서열을 탐지할 수 있는 Chromogenic *in situ* hybridization(CISH)을 그와 비교하여 평가하였다. 유속 세포 분석을 수행하기 위해 정자를 SYBR 14와 PI로 염색하였고, histogram, dotplot, density, contour를 측정하였다. Y 염색체 특이적인 primer를 이용한 PCR로 유속 세포 분석의 정확도를 검사하였다. HRP/DAB 시스템에 기초한 CISH 분석에는 X 또는 Y 염색체에 상보적으로 결합하는 probe가 사용되었다. CISH 분석은 기존의 방법들보다 빠르고 쉬우며 비용이 적게 든다는 장점이 있다. 또한, CISH는 보다 정확한 정자의 선별을 가능하게 하는 것으로 나타났다. 본 연구에 따르면 CISH가 기존의 선별 방법들을 평가하는 기술로서만이 아니라 특정한 성별을 가진 포유동물의 생산에도 사용될 수 있을 것이다.

(Key words : sex determination, boar sperm, chromogenic *in situ* hybridization, flow cytometry)

INTRODUCTION

The development of semen sexing is widely accepted as a major advance in reproductive technology. The sorting of the sex accelerates genetic progress as well as enhancing benefit the management and efficiency of mass production of livestock (Inmaculada *et al.*, 2005). Sorting of sex has been accomplished in several species of mammals such as bovine (Hamano *et al.*, 2007), chicken (Shimada *et al.*, 2007), pig (Rath *et al.*, 1997), cattle (Cran *et al.*, 1995), rabbit (Johnson *et al.*, 1989) and also in human (Fugger *et al.*, 1998) using flow-cytometric sperm sorting. This method is based on the measurement of the difference in the relative DNA content of X- and Y-bearing spermatozoa after staining with SYBR 14/PI (propidium iodide; Garner and Johnson, 1995). Offspring of a pre-determined sex have been produced using sex-sorted sperm after artificial insemination (AI; Johnson *et al.*, 1991), *in vitro* fertilization (IVF; Rath *et al.*, 1999; Abeydeera *et al.*, 1998) or intracytoplasmic sperm injection (ICSI; Probst and Rath, 2003). However, the equipment for sexing sperm is fairly complicated and expensive over $350,000 per sperm sorter. It is also expensive to install and maintain. Skilled operators are required which results in training costs (Seidel Jr., 2007). An alternative for sex determination with conceptus or post-natal tissue is identification of sex chromosomes by employing fluorescent *in situ* hybridization (FISH; Wyrobek *et al.*, 2005) where complementary sequences on interphase X and Y chromosomes are labeled with multi-colored DNA probes. FISH can provide a more complete identification of gender than PCR-based techniques (Sato *et al.*, 2003; Gimenez *et al.*, 1994), but FISH may be difficult and fluorescence fades quickly. We suggest chromogenic *in situ* hybridization (CISH) as new methods for confirming FACS sperm sorting. CISH analysis detects biotin-labeled probes by using HRP/DAB system, and it

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[1] Equal contributors

† Correspondence : E-mail : davekim@gachon.ac.kr

can de conducted through easy and less expensive. CISH has been used to analyze HER-2 amplification or over-expression in breast cancer method (Vocaturo *et al.*, 2007; Tanner *et al.*, 2000). Our study would be the first challenge to apply CISH on germ cell. This technique combine with ICSI, it can be a powerful tool products offspring of desired sex.

MATERIALS AND METHODS

1. Sperm Preparation for Flow Cytometric Sorting

Semen was supplied with local AI center (Darby GeneTics Inc., Anseong, Korea) and sperm concentration was determined with a hemocytometer. Semen was washed three times in Beltsville Thawing Solution (BTS; Johnson *et al.*, 1988) at 1,500 rpm for 10 min. After washing process, the concentration of sperm was 1.21×10^7 /mL. Sperm were suspended in BTS, added SYBR 14/PI (Invitrogen, Carlsbad, CA) and incubated at 37℃ for 15 min.

2. Flow Cytometric Sperm Sorting

The viable sperm-sorting procedure was essentially that as described earlier (Johnson *et al.*, 1989). Sperm was sorted by FACS Vantage SE (Becton Dickinson, Franklin Lakes, NJ) and Phosphate-buffered saline (PBS, Gibco BRL, Grand Island, NY) was used as sheath fluid. The sorting wave of used FACS machine was 351～364 nm and data were calculated by CellQuest software (BD Biosciences, San Jose, CA). After X, Y sorting process, each 300,000 sperms were collected in conical tube and transported to laboratory in adequate storage condition.

3. PCR and Gel Electrophoresis

Previously, we synthesized primer specific to chromosome 1 and Y chromosome respectively (Table 1). The PCR amplification was carried out in a programmable thermal cycler (MJ mini, Bio-Rad Laboratories, Inc. Mississauga ON). The amplification profile comprised 30 cycles: at 94℃ for 20 sec (denaturation), 60℃ for 10 sec (annealing) and 72℃ for 30 sec (extension). We performed gel electrophoresis (DNA; 5 μL, 1% agarose, TAE buffer). By comparing the difference of band strength, we confirmed sorting efficiency.

4. CISH

Chromosome X centromeric probe (Zymed, South San Francisco, CA) or Chromosome Y probe (Zymed) was added to semen on a slide. Coverslip was placed; prove side down, in the appropriate area of the semen on slide. Coverslip was sealed to prevent evaporation during incubation. 5 mL syringe containing rubber cement was used and topped with yellow tip. Rubber cement was carefully applied to the edges of the coverslip, slightly overlapping onto the slide. Rubber cement was dried for 20～30 min to prevent coverslip from sliding off slide. This sealing step was modified with adhesive tape. Tape had advantages easy peeling off and no drying step. Probe was denatured by incubating slides at 94～95℃ for 5 min. Slides incubated at 37℃ for 10 h. Two Coplin jars containing 0.5 × standard saline preheated citrate solution (SSC; Zymed) buffer were prepared, one at RT, the other heated to 75℃. After hybridization, rubber cement was carefully peeled off. Slides were rinsed briefly in the jar containing RT SSC, and then slides were immersed for 5 min. in the jar containing SSC at 75℃. Slides were washed in dH_2O. Slides were immersed in 3% H_2O_2 (Sigma) in Absolute Methanol (Sigma) for 10 min, and washed in PBS/Tween 20 (0.025%, Gibco/Sigma) 3 times for 2 min each. CAS-Block (Zymed) was added for 10 min. After clot off blocking reagent, HRP-Streptavidin (Zymed) was added for 30 min. Again slides were washed in PBS/Tween 20 (0.025%), and then DAB (Zymed) was added for 30 min. After hematoxylin counterstaining for 1 min, slides were washed with running tap water. Slides were dehydrated in graded EtOH

Table 1. Oligonucleotide primers specific chromosome Y and 1

Species	Primer name	Primer direction	Primer size(bp)	Primer sequence	Specific amplicon size	Reference
Sus scrofa (pig)	Ch Y	Forward	22	5'-AAT CCA CCA TAC CTC ATG GAC C-3'	377 bp	×12696
Sus scrofa (pig)	Ch Y	Reverse	20	5'-TTT CTC CTG TAT CCT CCT GC-3'	377 bp	×12696
Sus scrofa (pig)	Ch 1	Forward	22	5'-GTT GCA CTT TCA CGG ACG CAG C-3'	244 bp	×51555
Sus scrofa (pig)	Ch 1	Reverse	22	5'-CTA GCC CAT TGC TCG CCA TAG C-3'	244 bp	×51555

(Sigma) series, and immersed in Xylene (Zymed) 2 times for 2 min.

5. Microscopic Analysis of CISH Slides for Sex Determination

Sperm labeled X or Y chromosomes were scored on a ZEISS Axiovert 200 inverted microscope. Images were recorded without excitation and emission filters. Images were captured through Axiovision AC, and edited using Image J 1.38× program (NIH, USA). Sperm were scored as X or Y based on brown deposit in the sperm head under the bright field microscope (× 400).

RESULTS

Before FACS analysis, the result of SYBR 14/PI staining of boar sperm is shown in Fig. 1. Flow cytometric sperm sorting results are calculated in four forms of Histogram, Dotplot, Density and Contour (Fig. 2). Validation of flow cytometric sperm sorting was conducted by PCR. It showed FACS sorting is accurate about 90% (Fig. 3). CISH probes are a double-stranded DNA probe that has been labeled with biotin. Chromosome X probe binds specifically to the centromere of chromosome X, and chromosome Y probe binds to chromosome Y band q12. As shown in Fig. 4, probes were detected in sperm head leant to the acrosome. When negative and positive identifications were grouped together, a good agreement to expected 0.5 ratios of Y chromosomes to total chromosome was evidenced. In particular, 79 sperms were negative and 63 were positive with both probes. As summarized in Table 2, we concluded that the accuracy of sperm sorting increase by chromosome X probe rather than by Y probe. Moreover, we correlated concentration of probe and sex-identification results (Table 3). We could demonstrate the optimum concentration is 1:1 ratio. In conclusion, sperm sorting by CISH could be the most suitable using X probe in the ratio of 1:1.

Fig. 1. Boar sperm stained by SYBR 14/PI.

DISCUSSION

The current state-of-art in sexing of mammalian sperm for offspring production is presented. The method is based on the differential amount of DNA being present in the X- and Y-chromosome-bearing sperm which is used as a marker to sort the X and Y populations. DNA content measurements by FACS have provided the precision necessary to identify the X- and Y-chromosome bearing sperm populations in semen from at least 23 mammalian species; boar 3.6%, mouse 3.6% and baboon 4.2% (Johnson *et al.*, 2000; Maxwell *et al.*, 2004; Garner *et al.*, 2001; Seidel Jr *et al.*, 2002; Garner *et al.*, 2006). In this present study, we conducted flow cytometric sperm sorting after SYBR 14/PI staining. Through PCR, FACS technique was reanalyzed. Boar sperm was sorted for X or Y population at purities of 90% by FACS.

An alternative approach for sperm sorting is an identification of sex chromosome by FISH. Although FISH is in general considered the standard methodology to confirm sorting accuracy (Kawarasaki *et al.*, 1998), this methodology have some disadvantages as it requires expensive microscope equipment, morphological features are difficult to visualize, and fluorescence fades quickly, so it does not provide a permanent record.

To confirm sperm sorting, different techniques, such as the Quinacrine mustard staining for Y-chromosome bearing sperms identification (Ogawa *et al.*, 1988), the Quantitative Southern Blotting (Beckett *et al.*, 1989), the semi-quantitative PCR for Y- and X- chromosome detection, eventually associated with autoradiography analysis (Lobel *et al.*, 1993; Joerg *et al.*, 2004; Chandler JE *et al.*, 1998; Chandler JE *et al.*, 2002), the PCR analysis on single spermatozoa (Szyda *et al.*, 2000; Welch *et al.*, 1995) and the capillary electrophoresis (Checa *et al.*, 2002) have been developed. Nevertheless, all these kinds of approaches were often approximate and time-consuming (Parati *et al.*, 2006).

Previously CISH mainly studied to analyze HER-2 amplification of over expression in several types of cancer, which was introduced as an alternative to FISH (Vocaturo *et al.*, 2007). This work is the first report that applies CISH on germ

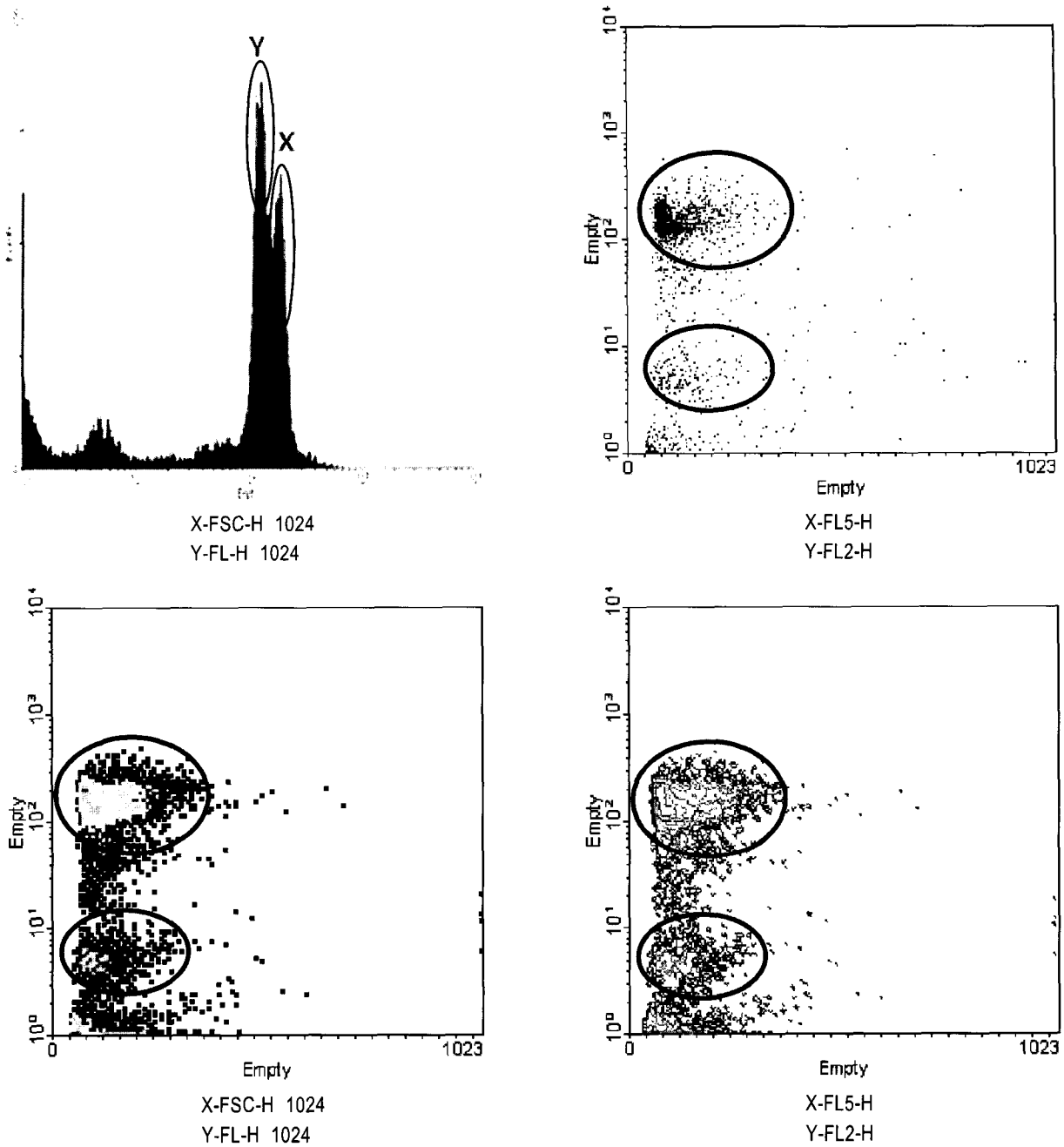

Fig. 2. FACS results through SYBR 14/PI staining.

cell. This study demonstrated a series of optimized methods for XY CISH sex chromosome-determination. The optimum probe concentration was 1:1 ratio, and chromosome X probe was more suitable to sort. Our study suggests that CISH can be applied to product offspring of desired sex, as well as to confirm flow cytometric sorted sperm. Sorted sperm using CISH can be used successfully for oocyte fertilization through intracytoplasmic sperm injection (ICSI).

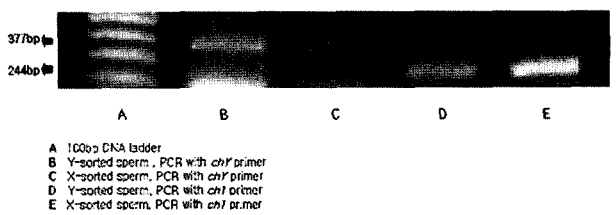

Fig. 3. Sorted sperm PCR using primer binding specific chromosome Y.

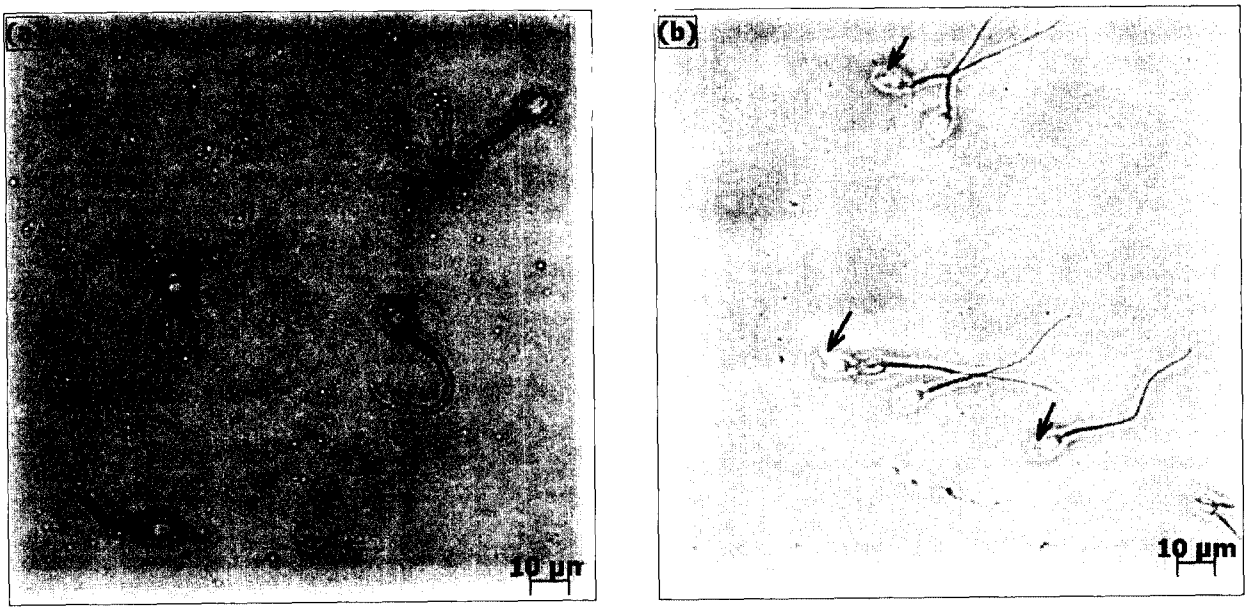

Fig. 4. Boar sperm samples analyzed through CISH (a); X-bearing sperm detected by chromosome Y probe (b) X-bearing sperm detected by chromosome X probe.

Table 2. Comparison between chromosome X and Y probe in CISH analysis

Probe	No identification (%)	Identification (%)	Total
Chromosome X	49 (53.8)	42 (46.2)	91
Chromosome Y	30 (58.8)	21 (41.2)	51
Total	79 (55.6)	63 (44.4)	142

Table 3. CISH results depends on probe concentration.

Sperm : Probe	No identification (%)	Identification (%)	Total
2 : 8	58 (47.9)	63 (52.1)	121
5 : 5	179 (49.7)	181 (50.3)	360
8 : 2	93 (39.4)	143 (60.6)	236
Total	330 (46.0)	387 (54.0)	717

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