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Applications of PCR and PRINS for the Sexing in Bovine Preimplantation Embryos

Seung Woon Seo, Hong Jun Lee, Ki Dong Kim, Sung Soo Park* and Sang Ho Lee

Departments of Amimal Science and Genetic Engineering, Korea Univ., Seoul 136-701*

착상전 소 초기배의 성판정을 위한 PCR과 PRINS의 적용

고려대학교 응용동물과학과, 유전공학과*

서승운 · 이홍준 · 김기동 · 박성수* · 이상호

= 국문초록 =

초기배의 성판정은 대상가축의 성을 선발하는 수단으로써 뿐만아니라 인간의 유전적 질병의 조기진단법으로서 매우 가치가 크다. 체외수정 소 초기배의 성을 결정하기 위해 PCR과 PRINS를 이용하였으며 성판정에 이용된 8 세포~배반포기 초기배는 체외수정후 난관상피세포와의 공배양에 의해 생산되었다. 초기배의 DNA는 200μg/ml proteinase K가 함유된 PCR lysis buffer에 하나의 초기배를 부유한 후 50℃에서 1시간동안 처리하여 준비하였다. 중기 염색체 spreads는 초기배를 nocodazole로 처리한 후 air-drying 방법을 이용하여 준비하였다. 가능한 false positive signals을 배제하기 위해 소특이 및 Y 염색체 특이 primers를 이용하여 PCR을 수행한 결과, 웅성 초기배에서는 두 개의 증폭산물(소특이 및 Y 염색체 특이)이 합성된 반면 자성 초기배에서는 하나의 증폭산물만 합성되었다. 한편 중기염색체상의 Y 염색체를 동정하기 위해 FISH와 PRINS를 수행한 결과, FISH에서보다 PRINS에서 더 강한 Y 염색체 특이 형광 signals이 탐지되었다. 이러한 결과는 PCR에 의한 체외생산 소 초기배의 신속정확하고 효율적인 성판정이 가능함을 보여주었다. 또한 PRINS를 통해 PCR에 이용된 Y 특이 probe의 신뢰성이 염색체 수준에서 확인되었다.

Key Words: PCR, PRINS, embryo, sex determination.

INTRODUCTION

Until recently, genetic diagnoses have been limited to the postimplantation period. Current advances in technologies comprising embryology and molecular biology have provided access to preimplantation embryos of many mammalian species. It has been suggested that diagnosis of genetic disease or at least genetic anomalies prior to implantation alleviates the psychological burden and the obstetric risk in human. Determination of the sex in preimplantation embryos is one approach to prevent the transmission of genetic anomalies. It also has a great value in the industry of animal production since it provides a means to select sex of interest.

It is well known that the sexual differentiation of mammalian embryos is directed by genetic element(s) located on the Y chromosome. Recently, species- and Y chromosome-specific DNA repeats have been identified and some sequences analysed in many mammals including mouse (Lamar & Palmer, 1984; Nallaseth & Dewey, 1986), human

(Cooke, 1976; Kunkel *et al.*, 1976), pig (McGraw *et al.*, 1988; Jeon *et al.*, 1994) and cattle (Leonard *et al.*, 1987; Reed *et al.*, 1988). These repetitive sequences have been used as molecular probes in determining sex of preimplantation embryos.

Embryo sexing can be carried out using the polymerase chain reaction (PCR) to amplify specific sequences present in the sex chromosomes, or by cytogenetic analyses such as fluorescent in situ hybridization (FISH) of specific probes to the X or Y chromosomes. Since Handyside *et al.* (1989, 1990) used amplification of a Y-specific repetitive sequence by PCR to sex human embryos, similar attempts have been made in other animals including mouse (Kunieda *et al.*, 1992), cattle (Peura *et al.*, 1991; Kirkpatrick & Monson, 1993) and pig (Pomp *et al.*, 1995). Although PCR is generally believed to be efficient and quick to obtain results, results can be plagued by contaminations. In the second, it is impossible to determine the number of sex chromosomes; thus, the same results would be obtained in cases of numerical chromosomal anomalies and normal males.

FISH allows direct visualization of DNA sequences of interest in metaphase or interphase nuclei. It has been successfully applied for sexing preimplantation embryos fertilized in vitro (Griffin *et al.*, 1992; Gimenez *et al.*, 1994; Bergere *et al.*, 1995). However, FISH failures are also possible, and they are more common in fetal cells than in adult cells (Grao *et al.*, 1993). As a result, the advantages and disadvantages of PCR and FISH for embryo sexing are still controversial. Recently, primed in situ DNA synthesis (PRINS) or in situ PCR has been developed to alternate previous sexing methods. These technologies have been shown to be capable of detecting low copy repeats which could not be shown by FISH (Koch *et al.*, 1989; Gosden *et al.*, 1991; Gosden & Lawson, 1994; Speel *et al.*, 1995).

In this report, we show a rapid sexing method by exploiting PCR. We also present PRINS with bovine Y-specific and autosomal probes to blastomeres of bovine preimplatation embryos to demonstrate reliability of embryo sexing at the chromosomal level.

MATERIALS AND METHODS

Preparation of embryonic DNA

Oocytes were collected from bovine ovaries, matured and fertilized in vitro as described elsewhere (Lee *et al.*, 1995). Embryos were developed to the eight-cell to blastocyst stages on a layer of bovine oviductal epithelial cells. To prevent possible contamination derived from sperm remnants following fertilization, zona pellucida was removed by consecutive treatments with 3% (w/v) sodium citrate and acid Tyrode's solution (pH 2.5). A zona-free embryo was washed in PBS containing BSA (4 mg/ml) and transferred into an Eppendorf tube containing 10 μl of lysis buffer (10mM Tris-HCl, pH 8.9, 1.5mM $MgCl_2$, 50mM Kcl 0.1%, w/w, Triton X-100, and 2% β-mercaptoethanol). After repeated freezing and thawing, embryos were treated with proteinase K (200 μg/ml) and used in following PCR reactions.

PCR amplification

Two sets of primers were used in this experiment. Nucleotide sequences were 5'-TGGAAGCAAAGAACCCCGCT-3' and 5'-TCGTGAGAAACCGCACACTG-3' of bovine 1.715 satellite DNA (Pluciennicczak *et al.*, 1982) for bovine-specific primers. Primers for Y-chromosome specific amplification were 5'-GGATCCGAGACACAGAACAGG-3' and 5'-GCTAATCCATCCATCCTATAG-3' (Reed *et al.*, 1988). The sizes of bovine- and Y-specific amplification products were 216 bp and 301 bp, respectively. PCR was carried out in a mixture containing DNA, 0.2mM dNTPs, 20-50 pmol primers, 1X PCR buffer (10mM Tris-HCl, pH 8.9, 50mM KCl, 1.5mM $MgCl_2$, 0.1% Triton X-100 and 0.01% BSA) and 2.5U Taq

DNA polymerase using a programmable thermal cycler. Each cycle consisted of denaturation at 94℃ for 30sec, annealing at 58℃ for 1min followed by extension at 72℃ for 1min. This was repeated 40 times. Finally, samples were held at 72℃ for 10min and cooled to 4℃. Amplification products were separated on a 2.5% (w/v) agarose gel, stained with ethidium bromide and visualized on a UV transilluminator.

Preparation of metaphase chromosome spread

The metaphase chromosome spreads were prepared according to methods described by Tarkowski (1966) and Kamiguchi *et al.* (1976) with slight modifications. Briefly, embryos were incubated in a CZB medium containing 15% FBS (Gibco BRL Co., USA) and 10 μM nocodazole for 16hrs at 36℃ under 5% CO_2, O_2 and 90% N_2. Subsequently, embryos were treated with hypotonic solution (1%, w/v, trisodium citrate) for 10 to 15min, transferred onto clean gelatin-coated slide and fixed in methanol:acetic acid (3:1).

Fluorescent in situ hybridization (FISH)

FISH was carried out by a method of Wiegant (1996) with minor modifications. Slides were washed in pre-warmed 2X SSC at 37℃ for 10min prior to dehydration through an ethanol series and air drying. DNA was denatured in 2X SSC containing 70% (v/v) formamide at 70℃ for 5min. Digoxygenin (DIG)-labelled probes were prepared by PCR reactions with DIG-11-dUTP as described above. Hybridization was performed in a solution containing 2X SSC, 1X Denhardt's solution, 5% (w/v) dextran sulfate, 100 μg/ml salmon sperm DNA and 400ng/ml DIG-labelled probe at 42℃ for 12hrs in a humid chamber. Post-hybridization washing was carried out 3 times with 2X SSC containing 60% formamide at room temperature for 5min each and once with a solution containing 10mM Tris-HCl, pH 7.5, 150mM NaCl and 0.05% (v/v) Tween 20 (TNT) for 5 min at room temperature. Finally, slides were incubated in a solution containing 100mM Tris-HCl, pH 7.5, 150mM NaCl and 0.05% blocking reagent (TNB) at 37℃ for 30min.

Detection of hybridization signals was performed using anti-DIG-Fluorescein (Boehringer Mannheim GmbH, Germany) according to supplier's specification. Anti-DIG-Fluorescein was diluted to 1:200 with TNB and layered on the slides. Samples were incubated at 37℃ for 1hr, washed 3 times with TNT, and dehydrated with ethanol series. Finally, slides were stained with 500ng/ml propidium iodide (Promega Co., USA) for 5min, washed with PBS for 10min and mounted with antifading solution.

Primed in situ DNA synthesis (PRINS)

For a rapid identification of Y chromosome-specific sequences on metaphase spreads, the PRINS reactions were carried out by methods described by Gosden and Lawson (1994) and Speel *et al.* (1995). Slides were treated with 0.5U T4 DNA ligase (Boehringer Mannheim GmbH, Germany) for 1hr at RT. The reaction was stopped by incubation for 5min in stop buffer (500mM NaCl/ 50mM EDTA) at RT. Chromosomal DNA and reaction mix were prepared as described for FISH. Forty microliters of reaction mix were layered on a denatured slide, and sealed with TaKaRa Slide Seal (Takara Shuzo Co.. Japan). Reactions were carried out on a Biometra thermal cycler programmed as annealing at 58℃ for 5min and extension at 70℃ for 20min. The reaction was stopped by transferring slides in stop buffer at 60℃ for 2min and the slides were washed with 4X SSC containing 0.05% Triton X-100 at RT. Finally, slides were incubated in 40 μl blocking buffer (4X SSC/ 0.05% Triton X-100/ 0.5% skimmed milk powder) at RT for 5min for subsequent detection. Detection was perfomed as for FISH described above.

RESULTS

Embryonic Sex Determination

A PCR amplification with two sets of bovine- and Y chromosome-specific primers was employed to determine the sex of embryonic samples. Bovine specific-sequences were amplified in both male and female, whereas only Y chromosome-specific sequences were amplified in male. As expected, amplified products of bovine- and Y-specific sequences were 216 bp and 301 bp, respectively. As shown in Fig. 1, two amplified products were obtained in male samples (lanes 2, 4, 5, 8, and 9), whereas only one product in female (lanes 3, 6, 7, and 10). The presence of a 216 bp bovine-specific product in all lanes and absence in the negative control (lane 1) indicate the success of the procedures. Same results were obtained with oocytes (results not shown); these confirming specificity of the Y-specific primers to male embryos.

A total of 92 embryos were successfully sexed by PCR and the results are summarized in Table 1. We also examined the sex ratio of the in vitro fertilized embryos. From a typical analysis, 41 (47.7%) of total 86 embryos were classified as male and 45 embryos (52.3%) as female, indicating that the sex ratio of sexed embryos did not differ significantly from the expected 1:1 ratio. PCR with parthenogenetic embryos produced bovine-specific product (female) alone.

Identification of Y chromosome on metaphase spreads by FISH and PRINS

FISH and PRINS allow direct visualization of DNA sequences of interest by detecting fluorescent signals on metaphase chromosome spreads or interphase nuclei. In order to identify Y chromosome-specific signals on metaphase spreads from bovine preimplantation embryos and to estimate efficiency and sensitivity of both methods, we have performed FISH and PRINS using bovine- and Y chromosome-specific probe, respectively. When FISH with DIG-labelled Y chromosome-specific probe was applied to the metaphase chromosome spreads prepared from bovine embryos, yellow-fluorescent signals were detected on the pericentric region of Y chromosome in the male (Fig 2, c), while no signals were detected on any chromosomes in the female. As shown in Fig. 2, with DIG-labelled bovine-specific probe, yellowish signals were detected on all chromosomes irrespective of male and female (Fig 2, a). The fluorescent signals were brighter and easily scorable in PRINS (Fig 2, b and d) than in ordinary FISH (Fig 2, a and c). The results also show that PRINS can increase the efficiency and reduce the time required for the sex determination and other preimplantation diagnoses of X-linked diseases in early embryos when comparing to FISH.

DISCUSSION

To be useful in determining the sex of

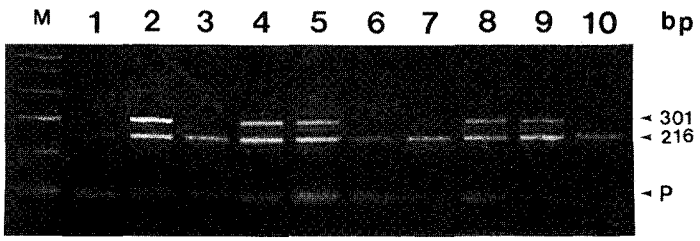

Fig. 1. PCR amplification of bovine embryonic DNA using bovine- and Y chromosome-specific primers. Lane M, DNA size marker; lane 1, PCR negative control: lanes 2 to 5, morulae embryos; lanes 6 to 10, 8- to 16 cell stage embryos; p, primers, respectively.

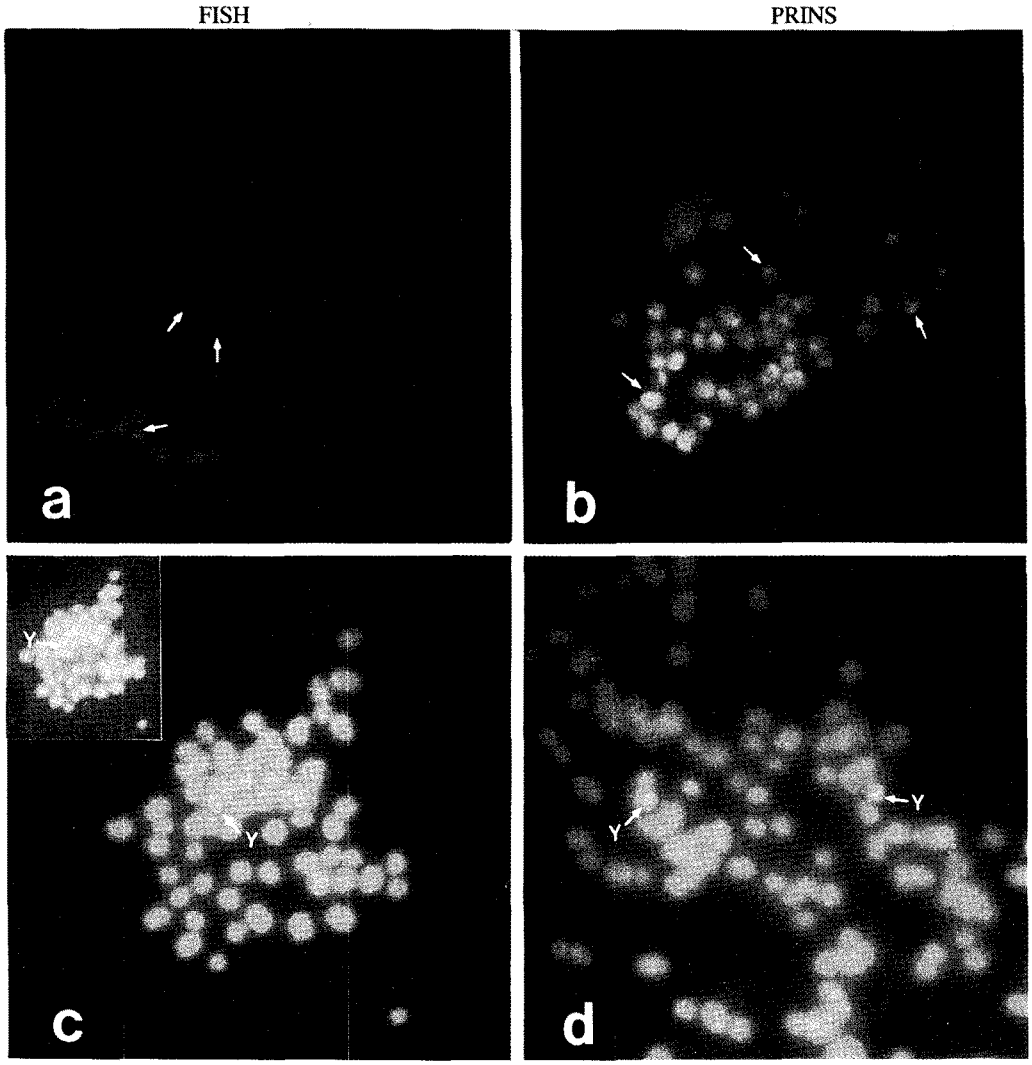

Fig. 2. FISH and PRINS to metaphase spreads from bovine embryos using bovine- (a and b) and Y chromosome-specific probe (c and d), respectively. A white arrow indicates positive signals (yellowish spots). Magnification was 1000x.

preimplantation embryos, any sexing technique must be totally reliable, simple, quick and efficient on a large number of embryos. Although PCR and FISH have been shown to be efficient and accurate methods for sex determination, their advantages and disadvantages are controversial as well as complimentary. In this study, PCR coupled either with FISH or with PRINS have been successfully applied in determining the sex of preimplantation embryos to improve the sex predetermination method developed previously (Seo *et al.*, 1995).

Recently, PCR has been used to determine embryonic sex in cattle (Peura *et al.*, 1991; Kirkpatrick & Monson, 1993), pigs (Pomp *et al.*, 1995), humans (Handyside *et al.*, 1989, 1990) and mice (Kunieda *et al.*, 1992) for the animal breeding programme and the diagnosis of X-linked disorders. Numerous studies have been carried out to determine sex in embryos using PCR that can amplify only single Y

Table 1. Representative sex predetermination of bovine preimplantation embryos by PCR

Source of embryos	Stages of embryos	No. of embryos	No. of embryos sexed as	
			Male (%)	Female (%)
In vitro fertilization	8~16-cell	34	16 (47.1)	18 (52.9)
	Morulae/Blastocyst	52	25 (48.1)	27 (51.9)
		86	41 (47.7)	45 (52.3)
Parthenogenesis	Blastocyst	8	0 (0.0)	8 (100)

*Total 199 embryos were analysed, 105 embryos were used in the preliminary experiments to set optimal PCR conditions.

chromosome-specific sequences. One of critical drawbacks with PCR is that failed reactions may lead to misclassification. To overcome this problem, autosomal gene amplification could be included in the parallel PCR reaction to verify the presence of DNA in the sample even in the absence of Y-specific signal (Peura *et al.*, 1991; Kunieda *et al.*, 1992). In our study, both bovine- and Y chromosome-specific primers were included in a single amplification reaction. Thus, incidence of misclassification became minimal, especially in the case of amplification failure due to inavailability of embryonic DNA. Secondly, sex determination of in vitro fertilized embryos by PCR can often be plagued by contaminating DNA derived mainly from sperm which are usually associated with zona pellucida. It was also found that positive amplification products could be generated by PCR with in vitro culture media (results no shown). Therefore, we included sodium citrate- and acid Tyrode's solution-treatments prior to embryonic DNA preparation. Using zona-free embryos could lead to more accurate determination of the sex in embryos. Other contamination sources, such as cumulus cells, can also be eliminated by the treatment.

FISH with X and Y chromosome-specific DNA probes has been mainly applied for sexing in vitro fertilized embryos for preimplantation diagnosis of X-linked recessive diseases and chromosomal abnormalities in the human (Griffin *et al.*, 1994; Bergere *et al.*, 1995) and mouse (Gimenez *et al.*, 1994). The freshness and quality of the chromosome preparations were of paramount importance for ensuring success. Optimal results are obtained with slides that are treated within a week of preparation.

The PRINS reaction, since its introduction (Koch *et al.*, 1989), has proved valuable for mapping and investigating repeated sequences in metaphase chromosomes or interphase nuclei, The technique has been steadily improved in both sensitivity and versatility (Gosden *et al.*, 1991; Gosden & Lawson, 1994; Speel *et al.*, 1995). The PRINS technique is based on the sequence specific annealing of an unlabelled oligonucleotide DNA in situ. This DNA serves as a primer for in situ chain elongation catalized by Taq DNA polymerase that uses labeled nucleotides and target chromosomal DNA as substrate and template, respectively. Since the strategy of in situ elongation of oligonucleotide primers has a number of advantages such as low background, high sensitivity and speed, PRINS technique provides a fast and reliable alternative to traditional FISH for chromosome screening. Since labelling occurs only after specific hybridization, background staining is very low. Secondly, the whole procedure including chain elongation and fluorescent detection is completed in a short span of time. Finally, target labelling is achieved with longer labelled probes which can improve sensitivity. Our results also confirms the superiority of PRINS

to FISH. Analysis of FISH results can often be hampered by low intensity of hybridization signals that is prominant where target DNA is present at low copy. This is also evidenced by our results which show more frequent and intense bovine-specific signals obtained by PRINS. In our results, the failure in detection of the Y-specific sequence by FISH could be mainly due to either loss or lack of a Y chromosome in embryonic cells occurred during metaphase spreading. However, this may not be the case since Y-specific signals were shown by PRINS. It is more possible that weak signals might have been resulted from low copy number of Y chromosome-specific probe. It has been shown that bovine Y chromosome-specific sequence used in our study is not nearly as repetitive as the bovine specific probe (1.715 satellite DNA) (Peura *et al.*, 1991).

In human, preimplantation genetic diagnosis may be an alternative to prenatal diagnosis for carriers of sex-linked disorders that have a 50% risk of transmitting the disease to male offspring. Previously, Horvat *et al.* (1993) employed a method of PCR co-amplification to identify the sex and presence of a microinjected transgene construct in bovine blastocyst embryos sectioned into quarters. Therefore, our results also implicate that sex predetermination using PCR and/or PRINS may be an efficient and accurate system in application to assisted reproduction procedures.

The results show that PRINS can increase the efficiency and reduce the time taken for the sex determination for preimplantation diagnosis of X-linked diseases in comparison with FISH.

It is also suggested that a rapid and accurate sexing is now possible in bovine preimplantation embryos using PCR. The reliability of this system was demonstrated by PRINS at the chromosomal level. In conclusion, PRINS may be a more desirable technique for sex determination than FISH in case of low copy repeats. Such a rapid system has obvious advantages for cytogenetic diagnosis.

CONCLUSION

Predetermination of sex in preimplantation embryos is of great value in the domestic animal production and clinical medicine since it provides a means to select animal sex of interest and diagnosis the human genetic diseases prior to implantation. Polymerase chain reaction (PCR) and primed in situ DNA synthesis (PRINS) were used to determine the sex of in vitro fertilized bovine embryos. Eight-cell to morulae stage embryos were obtained from in vitro fertilization (IVF) and subsequent coculture with oviductal epithelial cells. DNA was prepared by suspending single embryos in PCR lysis buffer containing 200 μg/ml proteinase K and incubated at 50℃ for 1hr. Metaphase chromosome spreads were prepared from nocodazole-treated embryos by air-drying method. To eliminate possible false positive signals, two sets of bovine- and Y chromosome-specific primers were used in the PCR. Two amplified products (bovine- and Y-specific) were obtained in male samples whereas only one product (bovine-specific) in female. FISH and PRINS were used to identify the Y chromosome on metaphase spreads. The fluorescent Y-specific signal was stronger in PRINS than in FISH.

The results suggest that a rapid, accurate and efficient sexing is now possible in bovine preimplantation embryos produced in vitro using PCR. This was evidenced by PRINS.

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