GENERAL INTRODUCTION

Recently, with the spread of technologies for the embryo transfer and the embryo manipulation, it has been possible to produce domestic animals which derived from embryos diagnosed on genetic traits. Several investigators have attempted to analyze genetic polymorphisms in preimplantation embryos, and one trait that has received much attention is sex of offspring. In domestic animals of the family Bovidae, sex preselection of offspring has been demanded for convenience of milk/beef production and animal breeding.

In cattle, cytogenetic analysis was initially attempted to identify sex of trophoblast biopsies from day 12 to 15 embryos (Hare *et al.,* 1976), bisected embryos (Picard *et al.,* 1984; Seike *et al.,* 1990), and biopsies from day 6 to 7 embryos (Schneider and Hahn, 1979; Singh and Hare, 1980). A limiting factor with the technique is the relative number of cells in metaphase. This means that large biopsies or pre-culture of the biopsy, which prevent effective use on a routine basis, are needed to obtain clear metaphase plates. Williams (1986) reported an embryo sexing method in mouse with assay of the X-linked enzyme, glucose 6-phosphate dehydrogenase (G6PD), which allowed the prediction of sex without embryo biopsy. This method has been followed the findings that female embryos have two X-chromosomes and both X-chromosomes seem to be potentially active in early embryos (Epstein, 1969). Embryo sexing with histocompatibility-Y (H-Y) antigen, presents in cells of the male, was also intensively studied in mice (Wachtel, 1984). These noninvasive sexing methods were also attempted in cattle. Iwata *et al.* (2002) showed that G6PD activity was high in female compared with that in male in morula stage embryos. Immunofluorescent detection (White *et al.,* 1987) and inhibition of blastocoel formation (Avery and Schmidt, 1989) using H-Y antibody were carried out in embryos. However, reproducibility and reliability of these noninvasive methods were insufficient for practical use. Individual differences in metabolism of embryos or quality of anti-H-Y antibody might cause the inconsistent results.

Identification of Y-chromosome specific DNA sequences enabled to develop molecular biological techniques for reliable embryo sexing. Fluorescence in situ hybridization (FISH) with DNA probe for Y-chromosome was used to distinguish between male and female cells (Cotinot *et al.,* 1991). To date, an accurate and rapid method by FISH has been reported whereas complicated process is needed (Kobayashi *et al.,* 2004).

Embryo sexing procedures based on Y-chromosome specific DNA amplification have also been reported in several species (human: Handyside *et al.,* 1989; mouse: Bradbury *et al.,* 1990; pig: Pomp *et al.,* 1995; sheep: Harpreet *et al.,* 2000) since polymerase chain reaction (PCR) was devised (Saiki *et al.,* 1985). In particular, PCR using highly repeated sequences on Y-chromosome made it possible to amplify the target sequence from a small number of blastomeres (Handyside *et al.,* 1989; Handyside *et al.,* 1990; Kageyama *et al.,* 2004). In cattle, Herr et al. (1990) reported the accurate sex prediction of calves by embryo sexing based on PCR. In comparison with earlier methods, PCR offered also the invaluable advantage of being so fast. In fact, PCR and electrophoresis can be accomplished within 3 to 5 h. This fact made it possible that sexed embryos could be transferred to a recipient without embryo cryopreservation. At present, embryo sexing systems with PCR has been employed for a commercial purpose in animal husbandry. However, PCR is not

an easy technology to perform embryo sexing in production fields, because strict thermal control is required for primer annealing and DNA synthesis. Furthermore, electrophoresis to visualize amplified products is time consuming and makes the risk of false positives because of DNA contamination. Therefore, development of an embryo sexing procedure that is more rapid and simple than that with PCR has now been demanded for the spread of the sex selection technology.

Embryo sexing in water buffaloes (*Bubalus bubalis*), which belong the different genus in the family Bovidae, has also been studied as well as in cattle (Appa Rao *et al.,* 1993; Manna *et al.,* 2003). Water buffaloes are important livestock as an alternative to cattle in hot-humid tropical climatic conditions, and have been bred for milk production especially in Europe. To date, although some Y-chromosome specific DNA sequences and sexing procedures with PCR for water buffaloes have been reported, the information is limited compared with bovine (Appa Rao and Totey, 1999; Horng *et al.,* 2004). Therefore, the analysis of Y-chromosome sequence in water buffaloes is important to develop an optimized sexing technique for water buffalo embryos.

Sensitive DNA amplification such as PCR proves promising clinical analysis method. In cattle, the freemartin, which is derived from heterosexual twin pregnancy and is congenitally sterile, could be diagnosed based on sex-chromosome chimerism in peripheral leukocytes (Padula, 2005). Cytogenetic analysis was used at first to detect XY leukocytes in heterosexual twin female calves (Ohno *et al.,* 1962; Kanagawa *et al.,* 1965a; Kanagawa *et al.,* 1965b). Y-chromosome specific DNA amplification by PCR was then utilized to find the presence of low frequent XY leukocytes (Olsaker *et al.,* 1993; Fujishiro *et al.,* 1995; Kadokawa *et al.,* 1995; Ennis *et al.,* 1999). Because early diagnosis of the freemartin is necessary for effective selection of fertile heifers, a rapid and simple procedure has been demanded as well as embryo sexing.

In contrast with PCR that a target DNA sequence is amplified by temperature change between about 50 and 95℃, isothermal DNA amplification methods have been recently developed. Loop-mediated isothermal amplification (LAMP) is a novel DNA amplification method that can amplify a specific DNA sequence within the range of 60 to 65℃ (Notomi *et al.,* 2000). DNA polymerase, with its high strand displacement activity, enables auto-cycling strand displacement DNA synthesis under isothermal condition. LAMP employs a set of four specific primers (termed inner and outer primer sets) that recognize a total of six distinct sequences on the target DNA (Figure 1A). Furthermore, an additional primer set (termed loop primers) is used to accelerate LAMP reaction (Nagamine *et al.,* 2002). An inner primer initiates primary DNA synthesis, and the following strand displacement DNA synthesis by an outer primer releases a single-stranded DNA derived from the inner primer (Figure 1B). The initial steps produce a stem-loop DNA structure, which is a characteristic DNA structure in LAMP, and then an extremely large amount of DNA is amplified from a stem-loop DNA by the autocycling reaction. In the fastest case, LAMP can amplify a target sequence within about 15 min. Furthermore, DNA amplification by LAMP can be detected by measurement of turbidity of a reaction solution, because a white precipitate of magnesium pyrophosphate (a by-product of DNA synthesis) is produced when a target sequence is successfully amplified (Mori *et al.,* 2001). Therefore, LAMP does not need electrophoresis to detect amplified DNA products. These properties indicate that LAMP would be suitable for field application of DNA analysis compared with PCR.

This study was performed to improve the efficiency of DNA diagnoses for embryo sexing and freemartin diagnosis using LAMP. In Chapter 1, a rapid

cattle embryo sexing method with LAMP was accomplished, and the reliability of the sexing method was verified by transferring sexed embryos to recipient animals. In Chapter 2, for sex chromosomal chimerism analysis in heterosexual twin female calves, the applicability of the LAMP-based embryo sexing procedure that was developed in Chapter 1 was evaluated. In Chapter 3, the homologues of cattle Y-chromosome specific DNA sequence in swamp and river buffaloes were cloned, and a rapid embryo sexing method for water buffaloes was accomplished using the DNA sequence and LAMP.

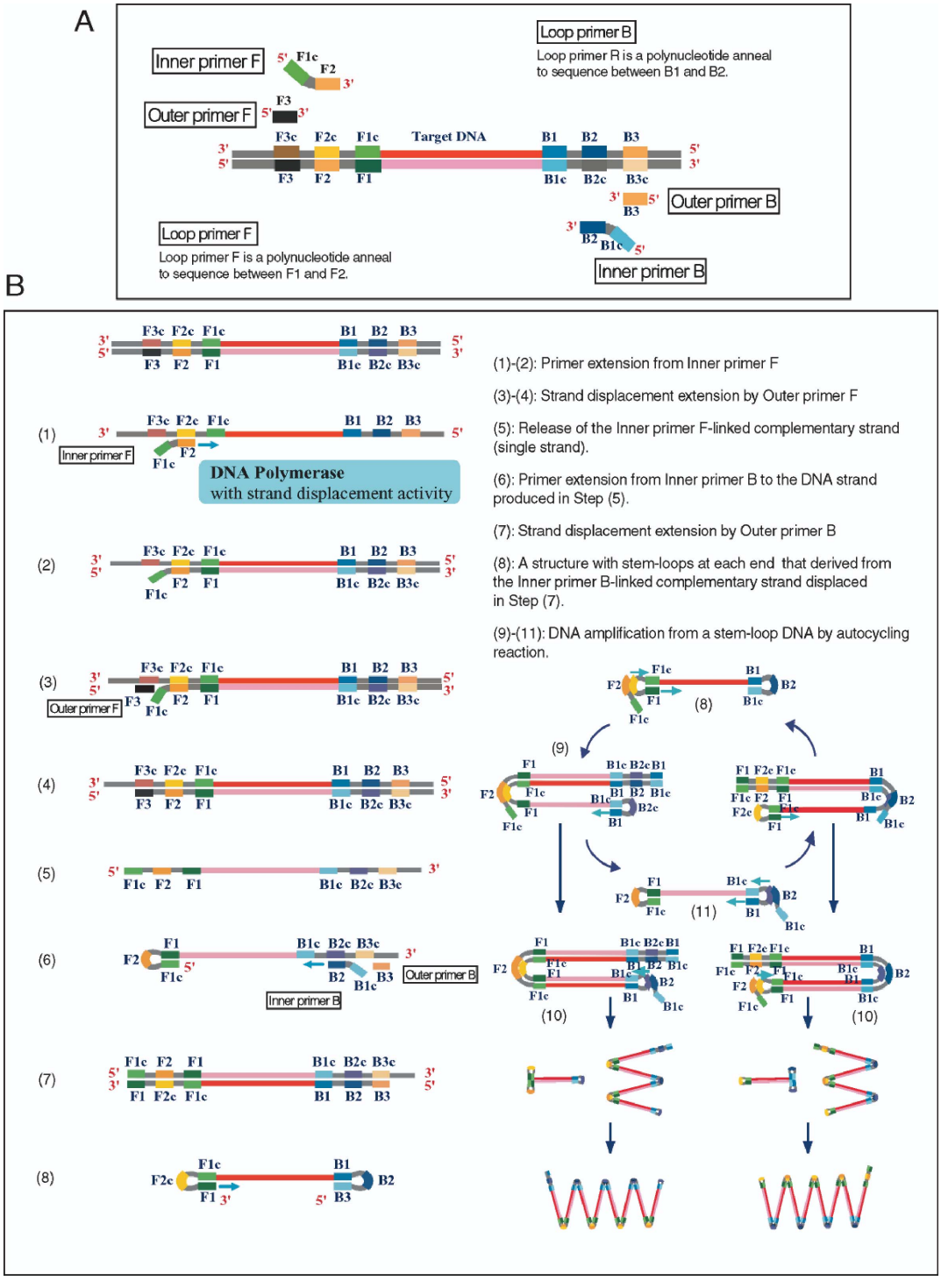

Figure 1. The principle of DNA amplification by LAMP. (A) Design of primers. (B) Basic principle.

Chapter 1

Sexing of cattle (*Bos taurus*) embryos using LAMP

Introduction

In the cattle embryo transfer industry, sexing of preimplantation embryos is an important management tool. Recently, sexing based on detection of Y chromosome-specific DNA sequences has been used to predict the sex of offspring. PCR (Thibier and Nibart, 1995; Chen *et al.,* 1999; Shea, 1999) including nested- (Greenlee *et al.,* 1998; Virta *et al.,* 2002) and primer-extension preamplification-PCR (Chrenek *et al.,* 2001; Garcia, 2001) is routinely used in the field for sexing; this technique enables amplification of a target sequence from a small number of blastomeres. However, these methods require technical skill and are time consuming. Furthermore, PCR has the risk of false positives because of DNA contamination during the handling of the PCR products in duplicate PCR procedures and/or electrophoresis. Therefore, for embryo sexing to become widely used in the cattle embryo transfer industry, a simple, rapid, and precise sexing method needs to be developed.

LAMP is a novel DNA amplification method developed by Notomi *et al.* (2000). The LAMP reaction is carried out under isothermal conditions (range, 60 to 65℃) using DNA polymerase with strand displacement activity. When the target DNA is amplified by LAMP, a white precipitate derived from magnesium pyrophosphate (a byproduct of the LAMP reaction), is observed. It is noteworthy that LAMP does not need special reagents or electrophoresis to detect the amplified DNA (Mori *et al.,* 2001). These properties of LAMP imply the applicability of LAMP to develop a rapid and precise procedure for embryo sexing with Y-chromosome specific DNA amplification.

The objective of the study in Chapter 1 was to establish efficient procedures for cattle (*Bos taurus*) preimplantation embryo sexing using LAMP. The author compared various DNA extraction methods, evaluated the sensitivity and the accuracy of the LAMP-based embryo sexing procedure, and transferred sexed embryos to recipient animals to verify the usefulness of the LAMP-based embryo sexing in the field.

Materials and methods

In vivo and *in vitro* embryo production

In vivo developed cattle embryos were obtained from the donor cows (Japanese black and Holstein) superovulated with follicle stimulating hormone (FSH) (20 IU for Japanese black, 24 to 50 IU for Holstein, Antorin®R・10, Denka Pharmaceutical Co., Ltd., Kanagawa, Japan) twice daily in decreasing doses over 3 days. An injection of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, cloprostenol 0.5 mg for Japanese black, Resipron®-C, Teikoku Hormone Mfg. Co., Ltd., Tokyo, Japan; tromethamine dinoprost 50 to 75 mg for Holstein, Pronalgon-F injection, Pharmacia, Tokyo, Japan) was given on the 3rd day of superovulation. Artificial insemination (AI) was performed 12 to 24 h after the onset of estrus and embryos were recovered 6 to 8 days after AI.

In vitro production of cattle embryos was performed as previously described (Takahashi and First, 1993; Takahashi *et al.,* 1996; Takahashi and Kanagawa, 1998a,b). Briefly, bovine oocytes col-

lected from slaughterhouse-derived ovaries were cultured for about 22 h at 39℃ under a humidified atmosphere of 5% CO_2 in air using a maturation medium (HEPES buffered TCM-199; Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS, Gibco), 0.02 units/ml of FSH (from porcine pituitary, Sigma Chemical Co., St. Louis, MO, USA), 1 mg/ml of estradiol-17β (Sigma), 0.2 mM sodium pyruvate, and 50 mg/ml of gentamicin sulfate. *In vitro* matured oocytes were co-incubated with frozen-thawed semen in modified Brackett and Oliphant medium containing 3 mg/ml of fatty acid-free bovine serum albumin (BSA, Sigma) and 2.5 mM theophylline (Sigma) for 18-19 h at 39℃ under 5% CO_2 and 5% O_2. After co-incubation with sperm, cumulus cells were removed from oocytes, and embryos were cultured for 6 days in modified synthetic oviduct fluid containing 20 amino acids (Sigma) and 10 mg/ml of insulin (Sigma) and further supplemented with 5 mM glycine, 5 mM taurine, 1 mM glucose, and 3 mg/ml of fatty acid-free BSA at 39℃ under 5% CO_2 and 5% O_2.

LAMP

The LAMP reaction was modified from the methods of Notomi *et al.* (2000), Mori *et al.* (2001), and Nagamine et al. (2002). The DNA was amplified at a constant temperature of 63.5 (Experiments 1 and 3) or 65℃ (Experiment 2) for 30 to 40 min in a 25 μl reaction mixture containing 1.6 μM inner primers, 0.2 μM outer primers, 0.8 μM loop primers, 1.4 mM dNTPs, 0.6 M Betaine, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)SO_4$, 8 mM $MgSO_4$, 0.1% Tween 20, 8 U *Bst* DNA polymerase (New England Biolabs Inc., Beverly, MA, USA), and 5 μl of DNA sample. The DNA fragments synthesized by the LAMP reaction were detected based on the production of a white precipitate of magnesium pyrophosphate(Figure 2). For endpoint turbidity measurement and real-time turbidity monitoring, the absorbance of the reaction mixture at 650 nm was measured. When the turbidity reached 0.1, the reaction was judged to be positive (Figure 3). The temperature control for the LAMP reaction and the turbidity measurement were performed using a Turbidimeter (Teramecs Co., Ltd., Kyoto, Japan) developed for DNA analysis by LAMP. Sexing was performed with male-specific and male-female common LAMP reaction. In all experiments, DNA sample extracted from biopsied cell with 10 μl extraction solution was divided equally into two tubes; one for male-specific reaction and the other for male-female common reaction. A male-specific sequence (S4) that was a tandem repeat sequence on the Y-chromosome (Kageyama *et al.,* 2004) was used for detection of male embryos. The male-female common reaction was performed using the 1.715 satellite DNA sequence (Pluciennicak *et al.,* 1982). The primer sequences are shown in Table 1. The loop primers, which accelerate DNA amplification by LAMP, were also used in each reaction (Nagamine *et al.,* 2002). When both of the reactions were positive, the sex of the embryos was judged as male. On the other hand, when only the common reaction was positive, the sex of the embryos was judged as female. No interpretation could be made in the absence of the positive common reaction.

Table 1.　Primer sequences for LAMP-based embryo sexing in cattle

Male specific primers	Inner primer F	5’-AGCTATGTGGCATGTGGATCCTTCCCT GGAAATGTTTAAGTG-3’
	Inner primer B	5’-TAAAGCCAGACACAGAGGTCACTTTT GCTTCTCTTTCCTGCTTC-3’
	Outer primer F	5’-AGCCAAGAAGTGGATGAATC-3’
	Outer primer B	5’-GCAGTGCATTTCCTCCTC-3’
	Loop primer F	5’-GGGATGGAAACTGTGCAT-3’
	Loop primer B	5’-ATTGCATGTGGAAGAACTGTAG-3’
Male-female common primers	Inner primer F	5’-GAGGAACATTGGCTTCTGGACAAGCT GGGGATTGCTCT-3’
	Inner primer B	5’-AGTGGAAGCAAAGAACCCCACCCAGT GAGCTCCAA-3’
	Outer primer F	5’-AGGCTGCCTCTTGTGTT-3’
	Outer primer B	5’-CATGGCCTAGAGACCAATC-3’
	Loop primer F	5’-CCTAGATGAGGTCTATTGGC-3’
	Loop primer B	5’-CTGCTCTCGAATTGTGACG-3’

Figure 2.　Detection of DNA fragments synthesized by LAMP reaction.　White precipitate of magnesium pyrophosphate yielded by LAMP reaction (+).　Negative

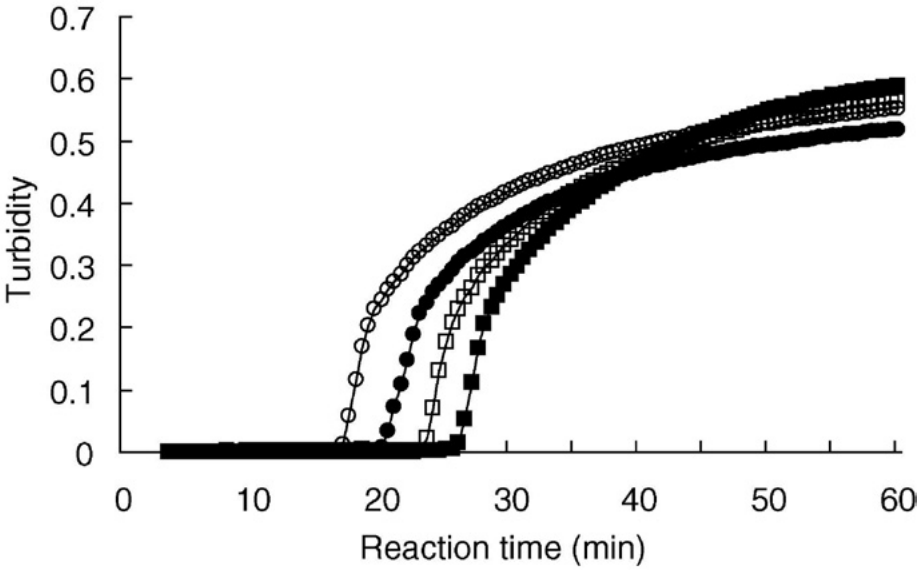

Figure 3. Real-time monitoring of DNA amplification using turbidity of LAMP reaction mixture. Absorbance of reaction mixture at 650 nm was measured every 30 sec for turbidity detection. Male-specific reactions using DNA extracted from single blastomere (●) and S4 (3000 copies) plasmid DNA (○) as template. Male-female common reactions using DNA extracted from single blastomere (■) and 1.715 satellite sequence (3×10^5 copies) plasmid DNA (□) as template.

PCR

The DNA of embryos was extracted by treatment with 5 μl of 10 mM Tris-HCl (pH 8.0) at 95℃ for 5 min and PCR was performed using HotStarTaq Master Mix Kit (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer's protocol. DNA was amplified in a 20 μl reaction mixture containing 0.75 μM primer and 5 μl of DNA sample. The primer set S4-b was used to amplify S4 (Kageyama *et al.,* 2004). The sequences of the S4-b primers were forward; 5'-CAAGTGCTGCAGAGGATGTGGAG-3' and reverse; 5' GAGTGAGATTTCTGGATCATATGGCTACT-3'. The reaction conditions consisted of initial denaturing at 95℃ for 15 min, followed by 15 cycles of 97℃ for 8 sec, 50℃ for 25 sec and 72℃ for 15 sec, and 30 cycles of shuttle PCR at 98° C for 8 sec and at 66 ℃ for 20 sec. The final extension step was followed by 5 min incubation at 72℃.

Experiment 1: DNA extraction methods

Zonae pellucidae of i*n vivo* -derived morulae were removed using a holding pipette and a glass needle attached to a micromanipulator. Blastomeres were separated by pipetting in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate buffered saline (PBS) supplemented with 1 mg/ml of polyvinyl-pyrrolidone (Sigma). Embryos that had been judged to be male based on PCR (with biopsies of 5 to 10 blastomeres) were used in the experiment. A single blastomere dissociated from a morula was treated with one of the following DNA extraction methods: (1) blastomeres were heated for 5 min at 95℃ in 10 μl of 10 mM Tris-HCl (pH 8.0) (heat method); (2) blastomeres were treated for 5 min at room temperature in 10 μl of 30 mM NaOH (NaOH method); (3) blastomeres were treated for 10 min at 37℃ in 10 μl of 0.1 mg/ml of proteinase K (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 0.5% Tween 20 (Nacalai Tesque, Inc., Kyoto, Japan). Proteinase K was then deactivated for 10 min at 99℃ (PK-TW method). Then, the LAMP procedure was used to determine sex of the embryos. Experiments were repeated three

times.

Experiment 2: sensitivity and accuracy of sexing by LAMP

In vivo - and in vitro-derived embryos were divided into 1 to 8 pieces using a microblade attached to a micromanipulator. The cell number in the piece was counted by staining with 0.5 mg/ml of Hoechst 33342 before sexing. The DNA from the biopsied cells was then extracted using the heat method. Sexing of DNA samples extracted from 1 to 5 biopsied cells was performed by LAMP. After biopsy, the remaining part of the embryos was used to confirm the sex by PCR. A total of 21 embryos (10 and 11 were judged as male and female embryos by PCR, respectively) were used.

Experiment 3: applicability of LAMP-based embryo sexing in the field

In vivo -derived embryos were biopsied using a microblade and subjected to determination of sex by LAMP. Two to twenty five cells(11.0 ± 5.1) obtained from the edge of morulae or the trophoblast of blastocysts were used as samples for sexing. The DNA of the biopsied cells was extracted using the heat method. Manipulated embryos were maintained for 3 to 4 h in IVD-101 medium(Research Institute for the Functional Peptides,Yamagata, Japan) at 39℃ under 5% CO_2 in air or in Dulbecco's PBS supplemented with 30% FCS at ambient temperature. Some manipulated embryos were transferred into recipient animals (18 heifers and 43 cows) 6 to 8 days after estrus. Forty-three of these cattle had estrus synchronized by the administration of a controlled internal drug release (CIDR) device for 7 to 14 days and injection of $PGF_{2\alpha}$ 1 day before removal of the CIDR device. Ultrasonographic pregnancy diagnosis was performed between 39 and 50 days after estrus. Some recipients in which estrus behavior was not observed by 60 days were assumed to be pregnant without pregnancy diagnosis. The accuracy of the sexing procedure was confirmed based on calf gender.

Statistical analysis

In Experiments 1 and 2, statistical differences between proportions reflecting various treatments and groups were evaluated by Fisher's exact test.

Results

Experiment 1: DNA extraction methods

As shown in Table 2, all DNA extraction methods showed high accuracies of sexing (89 to 94%), and there was no difference in accuracy among extraction methods (p >0.05).

Table 2. Effect of DNA extraction method on LAMP-based embryo sexing in cattle

Extraction method	No. of blastomere samples examined *	No. determined			Rate of correct determination (%)
		Male	Female	Unidentified	
Heat	18	16	2	0	89
NaOH	20	18	1	1	90
PK-TW	18	17	1	0	94

* A single blastomere was dissociated from morulae which had been judged as male by PCR.

Experiment 2: sensitivity and accuracy of sexing by LAMP

The detection of the male-female common reaction and the correct determination of sex were not different between *in vivo* - and *in vitro* -derived embryos and the data were combined as shown in Table 3. The common reaction was detected in 80 to 100% of samples, and the sex was correctly determined in 75 to 100% of samples. When sexing was performed using DNA samples extracted from 1 and 2 cells, the male-specific reaction was not detected in 3 samples derived from male embryos.

Table 3. Sensitivity and accuracy of LAMP-based embryo sexing in cattle

No. of blastomeres used for assay	No. of embryos examined	No. (%) with satellite sequence detected	No. (%) correctly determined
1	15	12 (80)	9 (75)
2	28	26 (93)	23 (88)
3	16	13 (81)	13 (100)
4	16	16 (100)	16 (100)
5	17	17 (100)	17 (100)

DNA of blastomeres was extracted with heat method (95 ℃ for 5 min).

Experiment 3: applicability of LAMP-based embryo sexing in the field

A total of 113 *in vivo* -derived embryos were subjected to the LAMP-based sexing; 58 (51%) and 55 (49%) of them were judged as males and females, respectively. Sixty-one of these fresh, sexed embryos, of which 23 and 38 were judged as male and female, respectively, were transferred to recipient animals (one embryo per animal). Thirty five (57%) of the recipient animals were diagnosed as pregnant, but two animals aborted, one at 83 and the other at 159 days after the transfer, as confirmed by ultrasound or rectal palpation. The remaining 33 recipient animals gave birth to 12 male and 21 female calves, all with the predicted sex.

Discussion

The removal of one or more cells from cattle embryos is essential for embryo sexing by the DNA amplification method. Biopsy is harmful to the embryo viability and reduces the pregnancy rate after embryo transfer (Thibier and Nibart, 1995; Hasler *et al.,* 2002). Therefore, the amplification of a target DNA sequence from a small number of cells is important for reducing the damage caused by embryo biopsy. Since an inadequate amount of template DNA causes failure of the amplification of a target sequence, DNA extraction methods for LAMP-based embryo sexing were examined. All three of the tested extraction methods showed adequate efficiency for single blastomeres. Proteinase K has been reported to be effective for extracting traces of DNA (Forell *et al.,* 2002; Virta *et al.* , 2002), but the procedure takes time and requires the deactivation of the enzyme. The present results indicate that heat and NaOH methods also have sufficient ability to extract DNA without enzyme treatment, and are suitable for LAMP-based embryo sexing. For subsequent experiments, the heat method was selected, since it is simple and rapid. LAMP-based embryo sexing system had high sensitivity and accuracy. The sex of embryos was determined correctly using 3- to 5-cell samples. As shown in Experiments 1 and 2, high sexing accuracy (75 to 94%) was also ob-

tained with 1- and 2-cell samples. Some male samples were misjudged as female samples in 1- and 2-cell sample groups; this implies that the sensitivity of the male-female common reaction is higher than that of the male-specific reaction. Many investigators have reported sexing efficiency of >90% and accuracy of about 90% in few or single blastomeres using the PCR technique (Machaty *et al.,* 1993; Bredbacka *et al.,* 1995; Shea, 1999; Chrenek *et al.,* 2001; Forell *et al.,* 2002; Park *et al.,* 2001). Therefore, the LAMP-based sexing procedure in the present study seemed to have sufficient sensitivity and accuracy for cattle embryo sexing.

Finally, the field application of LAMP-based embryo sexing was attempted. All tested embryos were sexed. The LAMP reaction required only 40 min and the total time for embryo sexing, including DNA extraction, was less than 1 h. After the transfer of sexed embryos to recipient animals, all calves born were of the predicted sex. Therefore, sexing by LAMP was a reliable method for use in the field. In this study, the transfer of *in vivo* embryos biopsied using a microblade resulted in a pregnancy rate of 57% . Thibier and Nibart (1995) reported that a higher pregnancy rate (55%) was achieved after transfer of embryos biopsied by aspiration compared with cutting (28%). Machaty *et al.* (1993) reported that a single blastomere biopsy did not influence the pregnancy rate (52.6%). However, using a microblade to perform biopsies is convenient and allows many embryos to be processed in a short time. Hasler *et al.* (2002) reported a 62% pregnancy rate for fresh embryos biopsied with a microblade (they removed 6 to 10 cells). Similarly, Shea (1999) also reported a 49% pregnancy rate after transfer of embryos from which 10 to 20% of the cells had been removed. The pregnancy rate obtained here seemed similar to previously reported results.

In this study, the target sequences were amplified within 40 min at both 63.5 and 65℃. The endpoint turbidity measurement and the real-time monitoring of the turbidity are convenient techniques for the detection of DNA amplification. Furthermore, the amplified DNA products are not removed from the reaction tube in this method, it helped to prevent contamination and to judge sex rapidly. Recently, sexing methods based on PCR without electrophoresis have been developed (Bredbacka *et al.,* 1995; Hasler *et al.,* 2002; Virta *et al.,* 2002). However, these methods require an ultraviolet transilluminator and expensive reaction equipment for rapid thermal cycling. Performing the LAMP reaction and measuring the resultant turbidity are extremely simple; LAMP requires equipment that furnishes a constant temperature and measures the turbidity of the reaction mixture (this equipment is inexpensive compared with PCR equipment).

In Chapter 1, the author developed a rapid and reliable cattle embryo sexing procedure based on LAMP. This technique makes it possible to easily determine the sex without thermal cycling or electrophoresis. Therefore, it is concluded that LAMP-based cattle embryo sexing is suitable for field applications.

Summary

LAMP is a novel DNA amplification method that amplifies a target sequence specifically under isothermal conditions. The product of LAMP is detected by the turbidity of the reaction mixture without electrophoresis. The objective of the study in this chapter was to develop a rapid sexing method for bovine preimplantation embryos using LAMP. The first experiment was conducted to optimize the DNA extraction method for LAMP-based embryo sexing. The DNA of single blastomeres was extracted using three methods: heat, NaOH and proteinase K-Tween 20 treatments. Sexing was performed with two LAMP reactions, male-specific and male-female common reaction, after DNA extraction. The rates of correct

determination of sex were 89 to 94% , with no difference among methods. The sensitivity and accuracy of LAMP-based embryo sexing were evaluated in the next experiment. The proportion of samples in which the sex was correctly determined was 75 to 100% for 1 to 5 biopsied cells. Lastly, in vivo-derived embryos were examined to verify the usefulness of LAMP-based embryo sexing, and some of these fresh, sexed embryos were transferred into recipient animals. The time needed for sexing was <1 h. The pregnancy rate was 57% and all calves born were of the predicted sex (12 male and 21 female). Therefore, LAMP-based cattle embryo sexing accurately determined gender and is suitable for field application.