

—Mini Review—

Sexing of Bovine Preimplantation Embryos using Loop-mediated Isothermal Amplification (LAMP)

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Abstract: Loop-mediated isothermal amplification (LAMP) is a novel DNA amplification method that amplifies a target sequence specifically under isothermal conditions. The LAMP product is detected by the turbidity of the reaction mixture without electrophoresis. We have developed a rapid sexing method for bovine preimplantation embryos using LAMP. Sexing is performed utilizing two LAMP reactions, male-specific and male–female common reactions, after DNA extraction. The time needed for sexing is <1 h. Sixty-one fresh sexed embryos, of which 23 and 38 had been judged as male and female, respectively, were transferred to recipient animals (one embryo per animal). The pregnancy rate was 57.4% (35/61) and all calves born were of the predicted sex. Therefore, LAMP-based embryo sexing accurately determined gender and was proven to be suitable for field application. In this review, we describe the procedure for detecting male-specific DNA sequence by LAMP for the rapid identification of freemartins. In addition, we also describe an efficient procedure for embryo sexing of water buffalo (*Bubalus bubalis*) with LAMP.

Key words: Sexing, Cattle, Embryo, LAMP, DNA amplification

Introduction

In the livestock industry, it is desirable to control the sex of animals. For example, dairy production benefits from the birth of female offspring. For meat production, male offspring are generally advantageous because male animals tend to grow faster and have better feed

efficiencies. In addition, the transfer of two embryos per female is sometimes conducted to double the efficiency of reproduction. If twin production is planned by the transfer of two embryos whose sexes are known before transfer, freemartin can be avoided. Therefore, the selection of the sex of embryos before transfer, according to a purpose, and knowledge of the sex of a fetus before parturition have high utility value. The sexing is expected to have a large effect not only on breeding improvement but also on economy.

In this review, we describe the development process of a bovine embryo sexing kit using LAMP and introduce some experimental data.

Prior to the Development of LAMP

Until the 1980's, a number of approaches were used to determine the sex of bovine embryos such as karyotyping [1, 2], H-Y antigen detection [3], and X-linked enzymatic determination [4, 5]. However, they failed to gain much popularity due to either a lack of accuracy or promptness [6]. In recent years, molecular genetics have developed, and the polymerase chain reaction (PCR) [7], which can amplify a DNA fragment of interest to generate a large number of copies of DNA, has been used in the field of animal husbandry to diagnose economic traits, disease, etc. The sex is economic trait in animal husbandry, and PCR has been used to determine the sex of bovine embryos [8–10].

We started to work on the development of a primer for sexing in 1991 and found a novel repeated DNA sequence specific for male cattle (named “S4”). We developed an embryo sexing method using a PCR primer set designed from the S4 sequence which was patented in 1997 [11]. The primer set amplifies a male-specific 178 bp product in addition to a 145 bp product common to both male and female cells. Although the origin of the 145 bp product is

unknown, it acts as a positive internal control in practical embryo sexing. Due to the high copy number (estimated at up to 1,000 copies) of S4, PCR requires only 0.5 pg of purified DNA for accurate amplification. This makes it possible to reduce the amount of biopsy sample required for embryo sexing, resulting in less damage to manipulated embryos. However, it is not easy to perform embryo sexing with PCR in the field, because strict thermal control is required for primer annealing and DNA synthesis. Furthermore, electrophoresis, which is needed for visualizing the amplified products, is time consuming, and carries the risk of false determination because of DNA contamination. Therefore, the development of an embryo sexing procedure that is more rapid and simpler than that of a conventional PCR has been demanded.

Loop-mediated Isothermal Amplification

Loop-mediated isothermal amplification (LAMP) is a novel DNA amplification method that can amplify a specific DNA sequence within a temperature range of 60 to 65°C [12]. DNA polymerase, with its high strand displacement activity, enables auto-cycling strand displacement DNA synthesis under an 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 (Fig. 1A). Furthermore, an additional primer set (termed loop primers) is used to accelerate the LAMP reaction [13]. 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 (Fig. 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 the turbidity of the reaction solution, because a white precipitate of magnesium pyrophosphate (a by-product of DNA synthesis) is produced when the target sequence is successfully amplified (Fig. 2) [14]. Therefore, LAMP does not need electrophoresis to detect amplified DNA products. These properties indicate that LAMP would be more suitable for DNA analysis in the field than PCR.

We developed a bovine embryo sexing kit by combining the patented male specific DNA sequence with the new DNA amplification technology, LAMP. This is the first case of the commercial use of the LAMP method, and feedback from PCR users is needed to improve our sex-

ing kit. Before starting the development (January 2001), a survey of the requests for the new sexing kit was conducted among organizations in Hokkaido using the PCR sexing method in the field. The strongest demand was for the prevention of false judgments of samples (determined to be female) because most embryo transfers are performed by private companies producing dairy cattle. Therefore, we aimed to develop a sexing kit using LAMP with a male–female common sequence in addition to a male-specific sequence which could distinguish between a false negative (failure to amplify the target sequence due to sampling error or reaction error) and a female.

A male-specific sequence (S4) that was a tandem repeat sequence on the Y-chromosome [11] was used for detection of male embryos. The male-female common reaction was performed using the 1.715 satellite DNA sequence [15]. The primer sequences are shown in Table 1. The loop primers, which accelerate DNA amplification by LAMP, are also used in each reaction [13]. When both of the reactions are positive, the sex of the embryos is judged to be male. On the other hand, when only the common reaction is positive, the sex of the embryos is judged to be female. No interpretation can be made in the absence of a positive common reaction.

Validation of the 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 of each 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.

The detection of the male-female common reaction and the correct determination of sex were not different between the *in vivo*- and *in vitro*-derived embryos and the data were combined as shown in Table 2. 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.

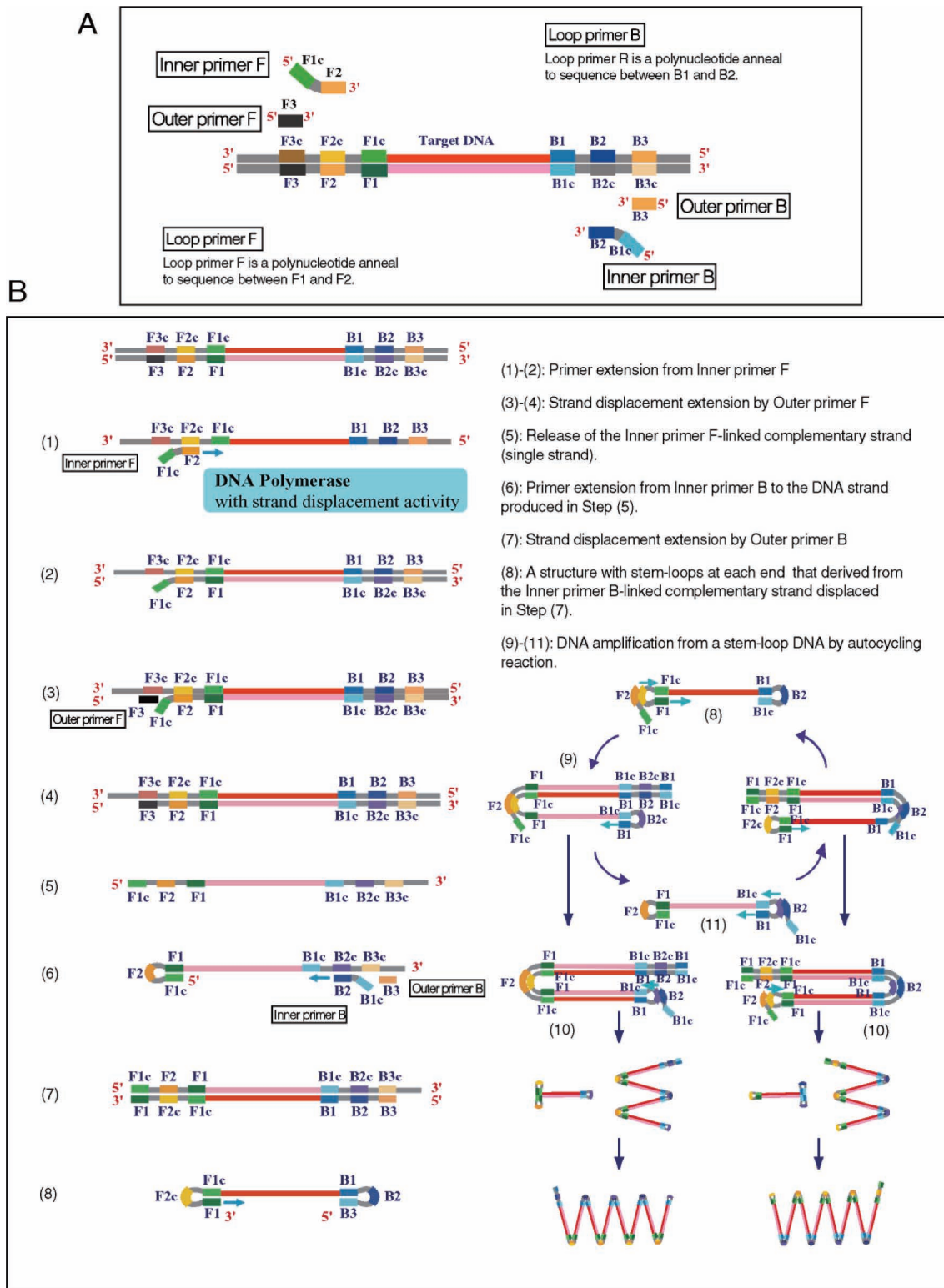


Fig. 1. The principle of DNA amplification by LAMP. (A) Design of primers. (B) Basic principle.

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

Male specific primers	Inner primer F	5'-AGCTATGTGGCATGTGGATCCTCCCTGGAAATGTTAAGTG-3'
	Inner primer B	5'-TAAAGCCAGACACAGAGGTCACCTTTGCTTCTCTTTCCTGCTTC-3'
	Outer primer F	5'-AGCCAAGAAGTGGATGAATC-3'
	Outer primer B	5'-GCAGTGCATTTCCCTCCTC-3'
	Loop primer F	5'-GGGATGGAAACTGTGCAT-3'
	Loop primer B	5'-ATTGCATGTGGAAGAAGTGTAG-3'
Male-female common primers	Inner primer F	5'-GAGGAACATTGGCTTCTGGACAAGCTGGGGATTGCTCT-3'
	Inner primer B	5'-AGTGGGAAGCAAAGAACCCACCCAGTGAGCTCCAA-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'

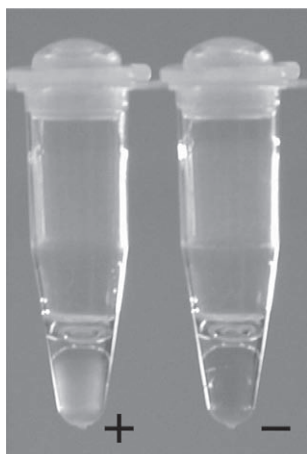


Fig. 2. Detection of DNA fragments synthesized in the LAMP reaction. White precipitate of magnesium pyrophosphate indicates the LAMP reaction is positive (+). Negative control (-).

Applicability of LAMP-based Embryo Sexing in the Field

In vivo-derived embryos were biopsied using a microblade (Fig. 3) 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. Some manipulated embryos were transferred into recipient animals (18 heifers and 43 cows) at 6 to 8 days after estrus. Ultrasonographic pregnancy diagnosis was performed between 39 and 50 days after estrus. The accuracy of the sexing procedure was confirmed based on calf gender.

A total of 113 *in vivo*-derived embryos were subjected to the LAMP-based sexing and 58 (51%) and 55 (49%) of them were judged to be males and females, respectively. Sixty-one of these fresh sexed embryos, of which 23 and 38 were judged to be 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 abortions were detected at 83 and 159 days of gestation by ultrasound or rectal palpation. The remaining 33 recipient animals gave birth to 12 male and 21 female calves, all with the predicted sex.

Application of the LAMP Sexing Method

The bovine freemartin, which is congenitally sterile, arises from the formation of a vascular connection between heterosexual twin fetuses during placental development [16]. In fact, about 90% of heifers, derived from heterosexual twin pregnancies, are infertile [17]. These calves are sold for meat or further rearing. However, if they are not recognized early, time can be wasted rearing them as potential replacements. The remaining female co-twins (about 10% of female calves born co-twin to male calves) are not freemartins. If all of the female co-twins were sold with no diagnosis, they would represent a loss of valuable genetic resources for milk production. An early diagnosis method for freemartins has therefore been demanded for preventing these losses.

The cattle embryo sexing procedure with LAMP was applied to sex chromosomal chimerism analysis of heterosexual twin female calves. The results showed the applicability of the embryo sexing procedure with LAMP for sex chromosomal chimerism analysis [18]. This procedure is useful for rapid determination of freemartins in heterosexual twin females. Therefore, instead of karyotyping and PCR, LAMP is recommended for identifying

Table 2. The sensitivity and accuracy of LAMP-based embryo sexing of 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 by the heat method (95°C for 5 min).

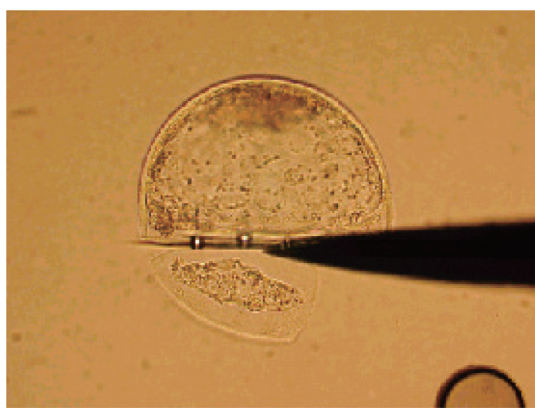


Fig. 3. Biopsy of a bovine embryo using a micromanipulator for sexing by LAMP.



Fig. 4. LAMP-based bovine embryo sexing kit.

freemartins, to prevent economic loss and the loss of important genetic material.

Water buffaloes (*Bubalus bubalis*), which are adapted to hot-humid tropical climatic conditions, are important livestock for multiple purposes (e.g. milk and meat production and farm power) in agricultural production. However, water buffaloes have low reproductive efficiency, and their production capacity has not been sufficiently utilized. If the LAMP sexing method for bovine embryos could be applied for water buffalo embryos, their reproductive efficiency would be improved. However, the procedure used to detect the Y-chromosome-specific sequence, S4, was found to be inapplicable to water buffaloes, perhaps due to genetic variation between these genera. Subsequently, we identified Y-chromosome-specific sequences in water buffaloes, and established an efficient procedure for water buffalo embryo sexing with LAMP [19]. This procedure can determine the sex of embryos derived from nuclear transfer using swamp buffalo fibroblasts as donor nuclei.

Conclusion

In this review, the authors have described the development of a field procedure for embryo sexing and sex chromosomal chimerism analysis, using a novel DNA amplification method, LAMP, which amplifies a target DNA sequence under an isothermal condition. Studies have showed that the LAMP method is very useful for DNA diagnoses using a small amount of template in the field.

Although the LAMP sexing kit (Fig. 4) has an almost 100% share of the market in Japan, there is a major issue with this embryo sexing method: low pregnancy rates after embryo transfer of frozen embryos which have been sexed (biopsied). In general, the pregnancy rate is about 50% after the transfer of fresh intact embryos, and about 45% after the transfer of frozen intact embryos. However, the pregnancy rate for biopsied embryos followed by freezing is very low, possibly because the biopsy reduces the embryos' tolerance to freezing. To solve this problem alternative cryopreservation methods such as vitrification are being tested. The simplification of the biopsy procedure is also an important issue. It is desirable to develop a new biopsy technique causing little or no decline in pregnancy rate or one which is easy to con-

duct under a stereomicroscope. The resolution of these issues would extend the range of application of embryo sexing in the field.

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