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Embryo Sexing and Sex Chromosomal Chimerism Analysis by Loop-Mediated Isothermal Amplification in Cattle and Water Buffaloes

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Abstract. In domestic animals of the family Bovidae, sex preselection of offspring has been demanded for convenience of milk/beef production and animal breeding. Development of the nonsurgical embryo transfer technique and sexing methods of preimplantation embryos made it possible. Sexing based on detection of Y chromosome-specific DNA sequences is considered the most reliable method to date. PCR enables amplification of a target sequence from a small number of blastomeres. However, it requires technical skill and is time consuming. Furthermore, PCR has the risk of false positives because of DNA contamination during 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. Loop-mediated isothermal amplification (LAMP) is a novel DNA amplification method, and the reaction is carried out under isothermal conditions (range, 60 to 65 C) using DNA polymerase with strand displacement activity. When the target DNA is amplified by LAMP, a white precipitate derived from magnesium pyrophosphate (a by-product of the LAMP reaction) is observed. It is noteworthy that LAMP does not need special reagents or electrophoresis to detect the amplified DNA. This review describes the development and application of an embryo sexing method using LAMP in cattle and water buffaloes.

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

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Today, with the spread of technologies for embryo transfer and embryo manipulation, it is possible to produce domestic animals derived from embryos diagnosed 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.

Double embryo transfer enables twining in cattle. Twins can, in most cases, increase the income from a single cow. The prediction of sex in preimplantation embryos is useful to prevent freemartins in heterosexual twins. Furthermore, genetic diagnosis technology with high sensitivity for embryo sexing is applicable to examination of sex chromosomal chimerism in heterosexual twin female calves.

This review describes the development and application of an embryo sexing method using an isothermal DNA amplification technique.

Embryo Sexing

In cattle, cytogenetic analysis was initially attempted to identify sex of trophoblast biopsies from day 12 to 15 embryos [1], bisected embryos [2, 3] and biopsies from day 6 to 7 embryos [4, 5]. A limiting factor with the technique is the relative number of cells in metaphase. Williams [6] reported an embryo sexing method in the mouse with assay of the X-linked enzyme, glucose 6-phosphate dehydrogenase (G6PD), which allowed the prediction of sex without embryo biopsy. This method was based on the findings that female embryos have two X-chromosomes and both X-chromosomes seem to be potentially active in early embryos [7]. Embryo sexing with histocompatibility-Y (H-Y) antigen, which is present in cells of the male, was also intensively studied in mice [8]. These noninvasive sexing methods were also attempted in cattle. Iwata *et al*. [9] showed that G6PD activity was high in females compared with males in morula stage embryos. Immunofluorescent detection [10] and inhibition of blastocoel formation [11] using H-Y antibody were carried out in embryos. However, reproducibility and reliability of these noninvasive methods were insufficient for practical use.

Identification of Y-chromosome-specific DNA sequences enabled to develop molecular biological techniques for reliable embryo sexing. Fluorescence *in situ* hybridization (FISH) with a DNA probe for Y-chromosome was used to distinguish between male and female cells [12]. To date, an accurate and rapid method using FISH has been reported; however, a complicated process is needed [13]. Embryo sexing procedures based on Y-chromosome-specific DNA amplification have also been reported in several species (human, Handyside *et al*. [14]; mouse, Bradbury *et al*. [15]; pig, Pomp *et al*. [16]; sheep, Harpreet *et al*. [17]) since polymerase chain reaction (PCR) was devised [18]. In particular, PCR using highly repeated sequences on the Y-chromosome made it possible to amplify the

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target sequence from a small number of blastomeres [14, 19, 20]. In cattle, Herr *et al*. [21] reported 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. This fact made it possible for sexed embryos to be transferred to a recipient without embryo cryopreservation. However, PCR is not an easy technology to use for 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 creates 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 is now needed for the spread of sex-selection technology.

Loop-mediated Isothermal Amplification

In contrast with PCR in which a target DNA sequence is amplified by a temperature change between about 50 and 95 C, isothermal DNA amplification methods have been recently developed. Loop-mediated isothermal amplification (LAMP) is a DNA amplification method that can amplify a specific DNA sequence within the range of 60 to 65 C [22]. DNA polymerase, with its high strand displacement activity, enables auto-cycling strand displacement DNA synthesis under isothermal conditions. 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 [23]. An inner primer initiates primary DNA synthesis, and the subsequent strand displacement DNA synthesis by an outer primer releases 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 the 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 [24]. 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.

Sexing of Cattle (*Bos taurus*) Embryos Using LAMP

Kageyama *et al*. [20] found a repeated DNA sequence designated S4 that is specific for male cattle. We developed a bovine embryo sexing kit by combining the S4 sequence with LAMP as the first case of the commercial use of LAMP (Fig. 2) [25]. The embryo sexing kit contains two kinds of reagents to detect the S4 sequence and the 1.715 satellite DNA sequence [25] from biopsied blastomeres. Detection of the 1.715 satellite DNA sequence makes it possible to confirm the absence of blastomeres in sample collection tubes, and thus false judgments of samples (determined to be female) are excluded. The embryo sexing kit has high sensitivity and can accurately determine sex of embryos from a few blastomeres (Table 1).

The removal of one or more cells from cattle embryos is essential for embryo sexing by the DNA amplification method. Biopsy is harmful to embryo viability and reduces the pregnancy rate after embryo transfer [26, 27]. Therefore, amplification of a target DNA sequence from a small number of cells is important for reducing the damage caused by embryo biopsy. Many investigators have reported sexing efficiency of >90% and accuracy of about 90% in few or single blastomeres using the PCR technique [28–33]. Therefore, the LAMP-based sexing procedure seemed to have sufficient sensitivity and accuracy for cattle embryo sexing.

Field application of LAMP-based embryo sexing has been attempted [25]. A total of 113 *in vivo*-derived embryos were subjected to 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, and 33 recipient animals gave birth to 12 male and 21 female calves, all with the predicted sex.

The embryo sexing kit amplifies the target sequences within 40 min at 65 C (Fig. 3). The endpoint turbidity measurement and real-time monitoring of the turbidity are convenient techniques for detection of DNA amplification. Furthermore, the amplified DNA products are not removed from the reaction tube in this method, which helps to prevent contamination and to judge sex rapidly. Although sexing methods based on PCR without electrophoresis have been developed [27, 29, 34], 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. Therefore, it is concluded that LAMP-based cattle embryo sexing is suitable for field applications.

Sexing of Water Buffalo (*Bubalus bubalis*) Embryos Using LAMP

Water buffaloes are important livestock as an alternative to cattle under hot-humid tropical climatic conditions, and have been bred for milk production especially in Europe. Embryo sexing in water buffaloes (*Bubalus bubalis*), which belong to a different genus in the family Bovidae, has also been studied as well as in cattle [35, 36]. We showed that LAMP has many advantages in field application of embryo sexing in cattle. However, the procedure used to detect a cattle Y-chromosome-specific sequence, S4 [20], is not applicable to water buffaloes, perhaps due to genetic variation between these genera.

A bovine Y-chromosome sequence, BRY, was identified as a repetitive sequence and has been reported to be conserved in sheep, goats, and deer [37, 38]. In water buffaloes, Appa Rao and Totey [39] reported a BRY.1 homologue sequence (BuRY.I) and developed a PCR procedure for embryo sexing using specific primers for BuRY.I. BRY.2 is a larger fragment of 3.7 kb, which was isolated as a male-specific sequence including a region homologous to BRY.1 [38]. We identified partial sequences of a BRY.2 homologue in swamp and river buffaloes that originated in regions different from BuRY.I and designated them sBuRY.2 and rBuRY.2, respectively [40]. BuRY.2 was highly conserved between swamp and river buffaloes, despite the fact that these species have different karyotypes (swamp type,

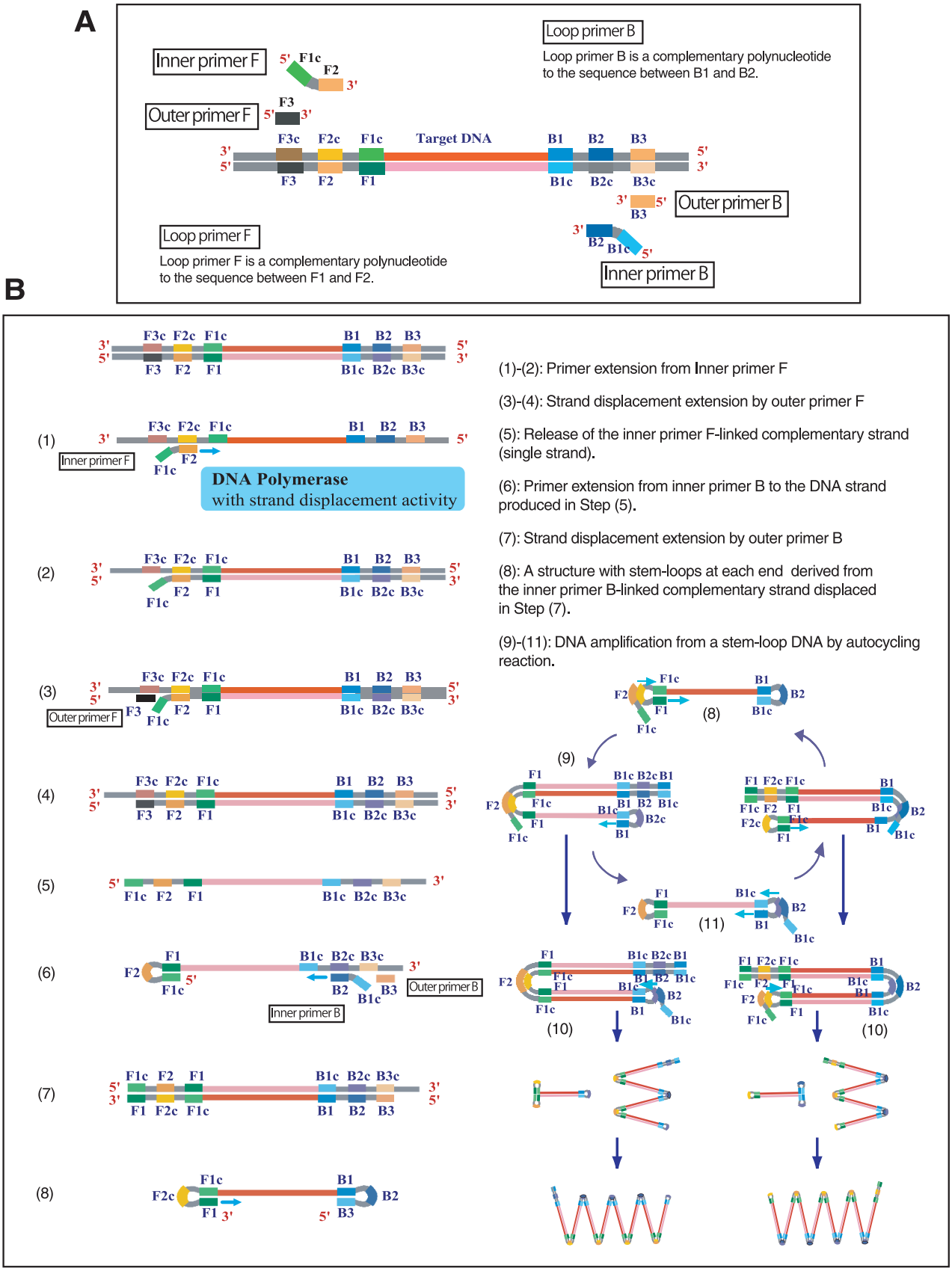

Fig. 1. The principles of DNA amplification by LAMP. (A) Design of primers. (B) Basic principle.

Fig. 2. Bovine embryo sexing kit with LAMP (A) and detection of DNA amplification by the turbidity of reaction mixture (B). The reaction tube on the left side in panel B shows the white precipitate of magnesium pyrophosphate yielded by LAMP reaction.

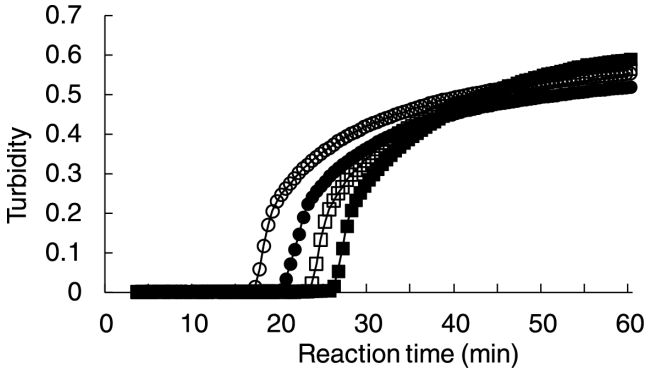

Fig. 3. Real-time monitoring of DNA amplification using the turbidity of the LAMP reaction mixture. Absorbance of the reaction mixture at 650 nm was measured every 30 sec for turbidity detection. Male-specific reactions using DNA extracted from a single blastomere (●) and S4 (3000 copies) plasmid DNA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

2n=48; river type, 2n=50) and have significant genetic differentiation [41, 42]. Therefore, the authors inferred that BRY-like sequences in the family Bovidae have remained stable.

Both male-specific (sBuRY.2) and male-female common (12S rRNA) DNA was detected by LAMP [40]. The LAMP reaction required only about 45 min, and the total time required for embryo sexing, including DNA extraction, was about 1 h. The procedure made it possible to determine the sex in a short amount of time compared with PCR-based procedures [35, 39]. The LAMP-based embryo sexing procedure showed sufficient sensitivity and accuracy when blastomeres of interspecies nuclear transfer (NT) embryos were reconstructed with nuclei of swamp buffalo and cattle oocytes. The sex determined from the blastomeres corresponded with the sex of the nuclear donor cells used for NT when the analyses were performed using 4 or 5 blastomeres as the templates.

Table 1. 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)

The applicability of LAMP for embryo sexing was shown in domestic animals other than cattle. We believe that this procedure will enhance the production efficiency of animals and promote animal breeding.

Sex Chromosomal Chimerism Aanalysis in Heterosexual Twin Female Calves by LAMP

The bovine freemartin, which is congenitally sterile, arises from vascular connection formation between heterosexual twin fetuses during placental development [43]. It has been reported that freemartins showed sex chromosomal chimerism in peripheral leukocytes [44, 45], and heifers with sex chromosomal chimerism presented abnormal development of the reproductive tract and poor ovarian response to gonadotropin administration [46, 47]. In fact, about 90% of heifers derived from heterosexual twin pregnancy are infertile [48]. These calves are sold for meat and further rearing. However, if they are not recognized early, it can be a waste of time rearing them as potential replacements. The remaining female co-twins (about 10%) are not freemartins. If all of the female co-twins are sold, they represent a loss of important genetic material. An early diagnosis method for freemartins has therefore been demanded for the preventing these losses.

Table 2. Cytogenetic and DNA analysis of chromosomal chimerism in peripheral blood

Animal			Sex chromosome analysis			DNA analysis	
Number	Age (weeks)	Vaginal length (cm)	60 XX	60 XY	(XY %)	PCR	LAMP
1	1	5	10	8	(44)	Chimera	Chimera
2	1	6	12	13	(52)	Chimera	Chimera
3	1	6	147	53	(27)	Chimera	Chimera
4	5	7.5	167	33	(17)	Chimera	Chimera
5	2	8	154	46	(23)	Chimera	Chimera
6	1	9	74	186	(72)	Chimera	Chimera
7	3	9	37	10	(21)	Chimera	Chimera
8	2	9	171	29	(15)	Chimera	Chimera
9	10	10	161	39	(20)	Chimera	Chimera
10	1	11.5	153	32	(17)	Chimera	Chimera
11	1	9	200	0	(0)	Normal	Normal
12	3	9.5	200	0	(0)	Normal	Normal
13	3	12	200	0	(0)	Normal	Normal
14	2	NE	200	0	(0)	Normal	Normal

NE, not examined.

To date, many freemartin diagnostic methods have been developed, for example, measurement of vaginal length [49], karyotyping [44, 45] and male-specific DNA amplification [50–53]. Male-specific DNA amplification methods based on PCR have particularly been used for sex chromosomal chimerism analysis in peripheral leukocytes because of their high sensitivity and rapidity.

Most (approximately 80%) freemartins will be correctly identified when the vaginal length test is used [49, 54]. It has therefore been proposed that obvious freemartins be identified by use of the vaginal length test and that the remaining clinically questionable calves be differentiated cytogenetically [55]. Miyake *et al*. [49] reported that karyotyping enabled the detection of sex chromosomal chimerism in heifers whose external genitalia were almost normal in appearance. However, karyotyping is time consuming, and it is difficult to determine the presence of a low XY leukocyte count of less than 1%. In contrast, chromosomal chimerism analysis by PCR amplifies with high sensitivity male-specific DNA in peripheral leukocytes, while the population of XY leukocytes to XX leukocytes cannot be estimated. Previous reports have shown that PCR amplification of Y-chromosome-specific repetitive sequences allowed the detection of 0.1 to 0.05% male blood in female blood [50, 53].

Chromosomal chimerism analysis in which a highly repetitive Y-chromosome-specific sequence was amplified by LAMP, also detected the existence of 0.01% XY leukocytes in female blood. The result indicates that this procedure is able to detect down to a level of 1/10000 XY leukocytes and has the same or higher sensitivity than PCR-based procedures. The accuracy of sex chromosomal chimerism analysis by LAMP was verified with samples from heterosexual twin females (Table 2). In all examined calves, there was no contradiction among results by karyotyping, PCR and LAMP analysis. To date, it has been confirmed that four non-chimeric heifers showed estrus and gave birth after AI.

Conclusion

Recently, the use of sex-sorted sperm has spread most rapidly in reproductive management of dairy cattle. On the other hand, embryo sexing is an important technique in animal breeding based on production of genetically selected bulls and dams. This review described that LAMP is very useful for DNA diagnoses using a small amount of templates in the field. We suppose that LAMP makes farm management more efficient through the spread of DNA diagnoses such as embryo sexing and sex chromosomal chimerism analysis.

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