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A rapid method for sexing the bovine embryo

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ABSTRACT

This study aimed to detect the sex of bovine embryo by adding ethidium bromide (EB) or 5 μl of $CuSO_4$ at different concentrations (0.5 M, 1 M, 1.5 M, 2 M, 3 M and 5 M) to the product of loop-mediated isothermal amplification (LAMP) reaction. The result of these additions was a colour change and a precipitate. This allows detection with the naked eye without the use of electrophoresis or a turbidity meter. The *in vitro* produced bovine embryos were divided into one to eight pieces using a microblade attached to a micromanipulator. The cell number in each piece was counted before sexing. Sexing of DNA samples extracted from one to five biopsies cells was performed by LAMP. After biopsy, the remaining part of the embryos was used to confirm the sex by polymerase chain reaction (PCR). The time for the whole procedure was about 45 min. LAMP is a novel DNA amplification method which amplifies a target sequence specifically under isothermal condition. We used this method to examine 58 bovine embryos and the accuracy of sex prediction was 100% when the blastomeres dissociated from a morula exceeds three. This study showed that the present method can be applied in bovine breeding programs to facilitate manipulation of the sex ratio of offspring. The aim of this study is to develop an improved embryo sexing technique based on the LAMP reaction. This new method is economic and more suitable for field application without turbidity meter or electrophoresis.

1. Introduction

Determination of the bovine offspring sex is very important for livestock farming and genetic improvement in cattle. Embryo sexing based on detection of Y chromosome-specific sequences has been used to predict the sex of the offspring (Alves et al., 2006) and polymerase chain reaction (PCR) including nested- and primer-extension preamplification-PCR enables amplification of a target sequence from a small number of blastomeres (Thibier and Nibart, 1995; Chen et al., 1999; Shea, 1999; Chrenek et al., 2001; Garcia, 2001; Virta et al., 2002). However, these methods require high levels of technical skills and are time consuming and PCR has a 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 bovine embryo transfer industry, a simple rapid and precise sexing method needs to be developed.

A rapid bovine embryo sexing procedure employing loop-mediated isothermal amplification (LAMP) has been reported by Hirayama et al. (2004). The feature of LAMP is specific DNA amplification under isothermal conditions. DNA polymerase, with its high strand displacement activity, enables auto-cycling strand displacement DNA synthesis within the range of 60–65 °C (Notomi et al., 2000). 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. 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

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displacement DNA synthesis by an outer primer releases a single-stranded DNA derived from the inner primer. 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 auto-cycling reaction. Accordingly, a white precipitate of magnesium pyrophosphate (a by-product of DNA synthesis) is produced (Mori et al., 2001). Therefore, amplification of a target sequence can be judged by measurement the turbidity in the reaction solution. It is noteworthy that LAMP does not need special reagents or electrophoresis to detect the amplified DNA. But the temperature control for the LAMP reaction and the turbidity measurement were also performed using turbidity meter developed for DNA analysis by LAMP, because it is very difficult to judge with the naked eye (Zhang et al., 2009).

The aim of the present study was to improve bovine embryo sexing techniques based on LAMP reactions reported by Hirayama et al. (2004) and Zhang et al. (2009).

2. Materials and methods

2.1. Embryo production assay

In vitro production of the embryos was performed as previously described by Takahashi and First (1993), Takahashi et al. (1996), Takahashi and Kanagawa (1998a,b) and Hirayama et al. (2004). Briefly, the bovine oocytes collected from slaughterhouse-derived ovaries and cultured for about 22 h under a humidified atmosphere of 5% CO_2 in air using a maturation medium (HEPES-buffered TCM-199) supplemented with 10% fetal calf serum (Gibco), 0.02 units/ml FSH (Sigma Chemical Co., St. Louis, MO, USA), 1 μg/ml estradiol-17β (Sigma), 0.2 mM sodium pyruvate, and 50 μg/ml gentamycin sulfate. Matured oocytes were co-incubated with frozen–thawed semen in modified Brackett and Oliphant medium (Takahashi and First, 1993) containing 3 mg/ml fatty acid-free BSA (Sigma) and 2.5 mM theophylline (Sigma) for 18–19 h at 38.5 °C under 5% CO_2 and 5% O_2 (Takahashi and Kanagawa, 1998a). After co-incubation with sperm, cumulus cells were removed from oocytes. Embryos were cultured for 6 days in modified synthetic oviduct fluid containing 20 amino acids (Sigma) and 10 μg/ml insulin (Sigma), 5 mM glycine, 5 mM taurine, 1 mM glucose, and 3 mg/ml fatty acid-free BSA at 38.5 °C under 5% CO_2 and 5% O_2.

In vitro, produced embryos were divided into one to eight pieces using a microblade attached to a micromanipulator. Cell number in the piece was counted by staining with 0.5 mg/ml Hoechst 33342 (Sigma) before sexing. DNA from the biopsy cells was then extracted. Sexing of DNA samples extracted from one to five biopsies cells was performed by LAMP. After biopsy, the remaining part of the embryos was used to confirm the sex by PCR (Kageyama et al., 2004). A total of 58 embryos were used in the present study.

2.2. DNA extraction assay

Zonae pellucidae of *in vitro*-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 PBS supplemented with 1 mg/ml polyvinyl-pyrrolidone (Sigma). The blastomere(s) dissociated from a morula was treated with the following DNA extraction method: blastomeres were heated for 5 min at 95 °C in 10 μl of 10 mM Tris–HCl (pH 8.0) as reported by Hirayama et al. (2004). Then, the LAMP procedure was used to determine sex of the embryo.

2.3. LAMP assay

LAMP reaction was preformed according to the method of Notomi et al. (2000), Mori et al. (2001), Nagamine et al. (2002), and Hirayama et al. (2004). DNA was amplified at a constant temperature of 65 °C for 30–40 min in a 25 μl reaction mixture containing 1.6 mM inner primers, 0.2 mM outer primers, 0.8 mM loop primers (Sigma), 1.4 mM dNTPs, 0.6 M betaine, 20 mM Tris–HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 0.1% Tween 20, 8 U Bst DNA polymerase and 5 μl DNA sample.

Sexing was performed with male-specific and male–female common LAMP reaction. DNA sample was extracted from biopsied cells with 10 μl extraction solution and divided equally into two tubes, one for male-specific reaction and the other for male–female common reaction. The male–female common reaction was performed using the 1.715 satellite DNA sequence (Nagamine et al., 2002 and Hirayama et al., 2004) as follows:

2.4. Male-specific primers

Inner primer F:
5′-AGCTATGTGGCATGTGGATCCTTCCCTGGAAATGTT-TAAGTG-3′
Inner primer B:
5′-TAAAGCCAGACACAGAGGTCACTTTTGCTTCTCTTTC-CTGCTTC-3′
Outer primer F:
5′-AGCCAAGAAGTGGATGAATC-3′
Outer primer B:
5′-GCAGTGCATTTCCTCCTC-3′
Loop primer F:
5′-GGGATGAAACTGTGCAT-3′
Loop primer B:
5′-ATTGCATGTGGAAGAACTGTAG-3′

2.5. Male–female common primers

Inner primer F:
5′-GAGGAACATTGGCTTCTGGACAAGCTGGGGATT-GCTCT-3′
Inner primer B:
5′-AGTGGAAGCAAAGAACCCCACCCAGTGAGCTCCAA-3′
Outer primer F:
5′-AGGCTGCCTCTTGTGTT-3′
Outer primer B:
5′CATGGCCTAGAGACCAATC-3′
Loop primer F:
5′-CCTAGATGAGGTCTATTGGC-3′