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Bovine embryo sex determination by multiplex loop-mediated isothermal amplification

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ABSTRACT

In cattle, the ability to determine the sex of embryos before embryo transfer is beneficial for increasing the number of animals with the desired sex. This study therefore developed a new modification of loop-mediated isothermal amplification in a multiplex format (multiplex LAMP) for highly efficient bovine embryo sexing. Two chromosomal regions, one specific for males (Y chromosome, S4 region) and the other common to both males and females (1.715 satellite DNA), were amplified in the same reaction tube. Each target was amplified by specifically designed inner primers, outer primers, and loop primers, where one of the S4 loop primers was labeled with the fluorescent dye 6-carboxyl-X-rhodamine (emitting a red color), whereas both satellite loop primers were labeled with the fluorescent dye fluorescein isothiocyanate (emitting a green color). After amplification at 63 °C for 1 hour, the amplified products were precipitated by a small volume of cationic polymer predispensed inside the reaction tube cap. Green precipitate indicated the presence of only control DNA without the Y chromosome, whereas orange precipitate indicated the presence of both target DNAs, enabling interpretation as female and male, respectively. Accuracy of the multiplex LAMP assay was evaluated using 46 bovine embryos with known sex (25 male and 21 female) generated by somatic cell nuclear transfer and confirmed by multiplex polymerase chain reaction. The multiplex LAMP showed 100% accuracy in identifying the actual sex of the embryos and provides a fast, simple, and cost-effective tool for bovine embryo sexing.

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1. Introduction

The ability to determine the sex of cattle embryos before transfer is useful for livestock management, particularly in the dairy cattle industry where female calves are preferred. The current methods for sexing bovine embryos normally rely on detecting Y chromosome–specific DNA using polymerase chain reaction (PCR) [1,2] or fluorescence *in situ* hybridization (FISH) [3]. However, these techniques are not widely used in the field as they are time consuming and labor intensive and require expensive instruments. Alternatively, use of a loop-mediated isothermal amplification (LAMP) technique for bovine embryo sexing has also been reported [4,5]. Loop-mediated isothermal amplification is a rapid isothermal DNA amplification technique, thus not requiring an expensive thermal cycler. Amplified products are subsequently detected on the basis of an increase in the turbidity of the reaction mixture, which can be assessed

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with a turbidimeter for accurate result reading. In embryo sexing, however, accurate result interpretation is challenging with turbidity measurements because they cannot differentiate the types of the amplified products. Therefore, two separate LAMP reactions, one for the male-specific detection and the other for the control reaction, have been required for complete bovine sexing. This inconvenience could result in DNA template loss and misidentification of the embryo's sex because the male-specific reaction is conducted in different reaction from the control. In this study, simultaneous detection of both male-specific DNA and control DNA in a single reaction tube using multiplex LAMP was developed to provide a simple, cost-effective, and accurate bovine sexing technique.

2. Materials and methods

2.1. Loop-mediated isothermal amplification primers

Two sets of LAMP primers (Table 1) were designed using the online LAMP Designer software (http://www.premierbiosoft.com/isothermal/lamp.html) and synthesized by Bio Basic Inc. (Markham, Ontario, Canada). Each set comprised two inner primers, two outer primers, and two loop primers. TK_S4, the primer set specific to male cattle, was designed from a highly repetitive sequence S4 (GenBank accession no. D16357) located on the bovine Y chromosome [6]. The other set, Sat1, specific to bovine DNA, was designed from the bovine 1.715 satellite DNA (GenBank accession no. V00125) [7] to serve as an internal control to indicate reaction validity. To differentiate the types of the amplified products, the TK_S4 backward loop primer was labeled with 6-carboxyl-X-rhodamine (ROX), emitting red color under ultraviolet (UV) light, and both Sat1 loop primers were labeled with fluorescein isothiocyanate (FITC), exhibiting green color under UV light.

2.2. Multiplex LAMP reaction

The multiplex LAMP was conducted in 25-μL reactions containing 1.4 mM dNTPs, 0.6 M betaine, 1X ThermoPol reaction buffer (New England Biolabs Inc., Beverly, MA, USA) supplemented with $MgSO_4$ to a final concentration of 8 mM, 8 U *Bst* DNA polymerase, the TK_S4 and Sat1 primer mix (1.6 μM of each inner primer, 0.2 μM of each outer primer, and 0.8 μM of each loop primer), and 5 μL of DNA template. Templates consisted of genomic DNA extracted from white blood cells of male or female cattle with the QIAamp DNA blood mini kit (Qiagen GmbH, Hilden, Germany) and quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). Nuclease-free water was used as the template in the negative control reactions.

The amplification reaction was carried out at a constant temperature of 63 °C for 1 hour in a mini dry bath (Benchmark Scientific, Inc., South Plainfield, NJ, USA), with the lid temperature set at 90 °C to prevent condensation of the reaction mixture on the lid. After amplification, the multiplex LAMP products were precipitated using polyethylenimine (PEI), and precipitate color was determined under UV light (365 nm). Orange precipitate indicated a male specimen because both the male-specific primers (red) and the internal control primers (green) were used and incorporated into the amplified products. On the other hand, green precipitate indicated a female specimen because only the internal control primers were incorporated in the amplified product, whereas the unused male-specific primers remained in the solution. A reaction without any precipitate was regarded as invalid, and no interpretation pertaining to the sex of the specimen could be made.

Table 1
Primer sequences for multiplex loop-mediated isothermal amplification.

Male-specific primers (TK_S4)
Inner primer forward: 5′-GTGGCATGTGGGATCTTAGTTCGGACTTCC CTGGAAATGT-3′
Inner primer backward: 5′-CCAGACACAGAGGTCACACAGCGTTACA GAATGCTTCTC -3′
Outer primer forward: 5′-CTATACAGCCAAGAAGTGGA-3′
Outer primer backward: 5′-CAGCCTTTATCAGAGCAGT-3′
Loop primer forward: 5′-GATGGAAACTGTGCATAGTCTT-3′
Loop primer backward: 5′-ROX-TGCATGTGGAAGAACTGTAGG-3′
Male–female common primers (Sat1)
Inner primer forward: 5′-ACTGAGGTTTTCCGGCACCCCGACCCTCCTG TCCAGAT-3′
Inner primer backward: 5′-TTCGGGGAACTTTGTGGGTCGAGGAGAAGTCCCACGTTAGG-3′
Outer primer forward: 5′-CCCCTTCCAGACAAAGCAG-3′
Outer primer backward: 5′-AGCCCTTTCCCGCTACTG-3′
Loop primer forward: 5′-FITC-TGAGCCCTTTCTCCCCTCCTG-3′
Loop primer backward: 5′-FITC-GTGCCAAGGGCCCTTTCGAC-3′

2.3. Polyethylenimine optimization

The amount of PEI for precipitation of multiplex LAMP products was optimized. A low-molecular-weight PEI (MW 600; Wako Pure Chemical Industries, Ltd., Osaka, Japan) was diluted to desired concentrations using sterile distilled water. A 2-μL droplet containing 0 to 10.8 μmol of PEI (calculated as monomer units) was predispensed inside each tube cap before the start of the reaction to avoid carryover contamination caused by opening reaction tubes after amplification. One nanogram of either male or female genomic DNA was used as the template, and nuclease-free water was used as a negative control. Equal concentrations of the TK_S4 and Sat1 primer mix were used for amplification. On completion of the LAMP reaction, reaction tubes were turned upside down to mix PEI droplets with the amplified products. Mixtures were then centrifuged briefly to facilitate amplicon precipitation. Precipitate color was inspected under UV light (365 nm), and the optimal amount of PEI was selected on the basis of precipitate size and the accuracy of precipitate colors in distinguishing sex.

2.4. Sensitivity and primer optimization

Two different concentrations of the primer mix were tested. In the first condition, equal concentrations of the TK_S4 and Sat1 primers were used, as described previously. In the second condition, a fivefold reduction of the Sat1

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