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A novel approach for determination of chicken sexing at an early stage of development by using loop-mediated isothermal amplification method

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Abstract: The monomorphism of newly hatched chicks is a major hurdle for poultry farmers at the time of sexing. The traditional sexing techniques are inaccurate and time-consuming. Therefore, the present study aimed to develop a loop-mediated isothermal amplification (LAMP)-based sex determination method to obtain a higher separation accuracy rate as compared to conventional methods. LAMP is a simple, quick, and relatively inexpensive diagnostic tool. Genomic DNA was extracted from the blood samples of Leghorn and Korean native chicks for LAMP. Two sets of primer were designed, consisting of four isothermal amplification primers in each, to amplify the female sex-specific sequence (HUR0417) of the *PKCIW* gene. We found that the corresponding female samples showed significant amplification of the *PKCIW* gene, while this was not observed in the male samples. Moreover, this method produced results in less than 1 h. The present investigation suggests that the LAMP-based sexing method is a sensitive, accurate, and time-saving method in chicks at an early age compared to the conventional sexing methods.

Key words: Chicken, *Bst* DNA polymerase, isothermal amplification primers, loop-mediated isothermal amplification, *PKCIW*, sex determination

1. Introduction

In recent years, knowledge of the molecular process underlying sex determination has significantly improved (1,2). Determination of sex holds great importance in animals; this is evident across species, ranging from simple eukaryotes to mammals. In many species, the sex of the individual is largely determined by the genes on one of the two sex chromosomes. In contrast, the molecular determinants of sex in avian species are the homogametic karyotype (ZZ), which corresponds to males, and the heterogametic karyotype (ZW), which corresponds to females. These processes have remained poorly understood (3–5). Most of the W chromosome does not recombine and a small pseudoautosomal region on Z and W obligates crossing-over during meiosis (2).

Chicken sexing is the method of distinguishing the sex of newly hatched chickens. The difficulty of sexing avian species stems from the absence of external sex organs in birds (6). Avian sex determination is a multiple gene regulation cascade. Genes such as the Z chromosome-linked *DMRT1* gene and W chromosome-linked *PKCIW* gene and other factors have been shown to be involved in this process (7). Recent studies on chicken embryos, carried out by microarray-based genome-wide gene profiling, demonstrated that a majority of genes located on the Z chromosome are not dosage-compensated and thus are expressed at a much higher level in males than in females (8,9). The lack of compensation of Z genes could lead to an increase in the expression of male determinants in ZZ individuals, leading to male sex determination. It was reported that a Z-linked double sex gene and mab-3-related transcription factor (DMRT1) are required for male gonadal sex determination (10). It was also proposed that the protein kinase C inhibitor (*PKCIW*) gene, which is located on the W chromosome, is involved in female sex determination (2,11,12). However, the functional significance of *PKCIW* in female sex determination remains unknown.

Sex separation of newly hatched chicks is an important aspect of the poultry industry. The varied segments of the

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poultry industry must sex chicks in order to segregate offspring for various reasons such as the implantation of two different feeding programs: the first program is for females (or hens) that are destined to lay eggs for commercial sale, and the other one is for males. Recently, there have been developments in the molecular sexing techniques based on DNA hybridization, where sex-specific sequences are detected in genomic DNA by complementary DNA probes. Microarrays are then used to compare the gene expression profiles of the primitive streak of individual male and female gastrulating chicken embryos. Polymerase chain reaction (PCR), in which sex-specific DNA is located by primers and then amplified, constitutes a breakthrough in the reliability and rapidity of sex identification in birds (13). The present conventional methods have certain drawbacks such as long detection period (3–4 h), expensive equipment, need for skilled technicians, and cost of chemicals and verification for conformation. These factors play an important role in the economy of the poultry industry and applicability in field conditions. Therefore, a simple, rapid, and cost-effective method for sex determination in chicks is needed. The present study was planned to develop a novel loop-mediated isothermal amplification (LAMP)-based method for chicken sexing at an early stage of development by using isothermal amplification primers. LAMP is a highly sensitive, cost-effective, one-step amplification reaction that amplifies a target DNA sequence with high sensitivity and specificity under isothermal conditions (14).

2. Materials and methods

2.1. Isolation of animal and genomic DNA

Leghorn layers of 35–40 weeks old were used in the present experiment after approval by the ethics committee of the National Institute of Animal Science, Namwon, Republic of Korea (Approval No: 2012 C-046 2012/03/02). A total of five repetitions were used to confirm and validate our findings.

To confirm the reactiveness of LAMP primers on known sex-determining DNA regions, approximately 1 mL of ulnar vein blood from a total of 100 chicks was collected in anticoagulant-coated tubes and stored at –20 °C. Genomic DNA was extracted from the whole blood by using a MagExtractor (TOYOBO, Japan) commercial DNA extraction kit. The DNA concentrations were determined using a NanoDrop 1000 Spectrophotometer (NanoDrop Technologies Inc., USA) and the samples were diluted to a final concentration of 10 ng/μL in nuclease-free water. To sex the developing embryos, 90 eggs (per treatments, primer sets A and B) were incubated at 37 °C and 65% relative humidity for 120 h in digital incubators (Rcom Max20, USA). Embryo fibroblast cells were separated from the 100 embryos at day 5 of development. The samples were placed into 1.5-mL microcentrifuge tubes and stored at –70 °C for DNA extraction.

2.2. LAMP primer design

Primer design is a crucial step for performing LAMP amplification. The isothermal amplification primers in LAMP consist of four primers: FIP (forward inner primer, corresponding to the F3 region at the 3′ end), F3 primer (forward outer primer, corresponding to the F3 region), BIP (backward inner primer, corresponding to the B3 region), and B3 primer (backward outer primer, corresponding to the B3 region). The target regions of the *PKCIW* gene sequence of W chromosome were amplified by two specific primer sets, having a total of four primers in each. Candidate LAMP primer sets, including F3, B3, FIP, and BIP, were designed by Explorer V4 (http://primerexplorer.jp/e/) by using default parameters. The primers were ordered from Cosmo Genetech, Korea. The primers' details are given in the Table.

Table. Isothermal amplification primers used in LAMP.

Sr. no	Name	Oligo	Primer
	1FIP	40 nt	5′-TTGTCCCCTAGCCCTTCCCTGAGCTGATCTTAGCACTGCG-3′
	1F3	19 nt	5′-CGAATTGGGGCTGGATGTG-3′
Set 1	1BIP	37 nt	5′-TTCGTTAAGTGGAAGGCTGCTGCCAGGCACAACTTCG-3′
	1B3	18 nt	5′-GGGAGCCAGCTTGAGAGA-3′
	2FIP	38 nt	5′-ATCACCACGGCCCCCAAGATTCATGTAGCAGGCGGGTC-3′
Set 2	2F3	19 nt	5′-CGGACTGGTGAGTGAACCT-3′
	2BIP	38 nt	5′-CTGTGCTCCCGTGGCCATACGCACATCCAGCCCCAATT-3′
	2B3	19 nt	5′-CCCGCAGTGCTAAGATCAG-3′

2.3. LAMP assay

LAMP was carried out in a total volume of 20 µL containing 1.5 µL of *Bst* DNA polymerase (New England BioLabs, USA) and a reaction mixture consisting of 2 µL of 10X reaction buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100), 0.6 µL (10 pM) of F3 and B3 primers each, 1.4 µL (10 pM) of FIP and BIP each, 2 µL of dNTP, and 2 µL (10 ng) of the genomic DNA. Each reaction was incubated at 65 °C for 1 h and then heated at 95 °C for 5 min to terminate the reaction by using a standard heat block or water bath.

2.4. LAMP product detection

LAMP amplicons in the reaction mixture were directly detected by three methods to analyze DNA amplification, the first of which included electrophoresis in 1.5% agarose gels stained with ethidium bromide (Sigma, USA). Second, for rapid sexing with the naked eye, direct visualizations in the reaction tubes were performed, with the addition of 1 µL of 10,000X RedSafe fluorescence dye (iNtRON biotechnology, Korea) in a 20-µL reaction mixture under a UV lamp. One sample was run as a negative control (primer-free) for both sets of primers in both methods. Third, enzymatic digestion was used to reconfirm that the target sequence was amplified; the repeated segment was digested with restriction enzymes and electrophoresed in 2% agarose gels, followed by staining with RedSafe fluorescence dye.

3. Results

The LAMP method relies on autocycling the strand-displacement DNA synthesis that was performed using the *Bst* DNA PCR, with specially designed inner and outer primers. In the present investigation, sex determination in chicks was confirmed by the absence of LAMP products in male chick samples. To select the specific primers for LAMP, we used two sets of primers.

A well-designed and optimized assay provides the highest specificity and yield. Special considerations should be made when performing assays to increase throughput when amplifying long templates. The specific temperature in the current investigation was optimized to 65 °C after performing the reactions at different temperatures ranging from 50 °C to 65 °C.

The current investigation was conducted to develop an uncomplicated, quick, and inexpensive technique to determine the sex in chicks by targeting the female-specific gene *PKCIW*. The results of gel electrophoresis showed the clear detection of the female-specific gene *PKCIW* in female DNA samples (Lanes 1–4 and 9–12) by both set 1 (Figure 1A, Lanes 1–4) and set 2 (Figure 1B, Lanes 9–12) isothermal primers. The male chicken samples (Lanes 5–7 and 13–15) did not show the target amplicon band by set 1 (Figure 1A, Lanes 5–7) and set 2 (Figure 1B, Lane 13–15)

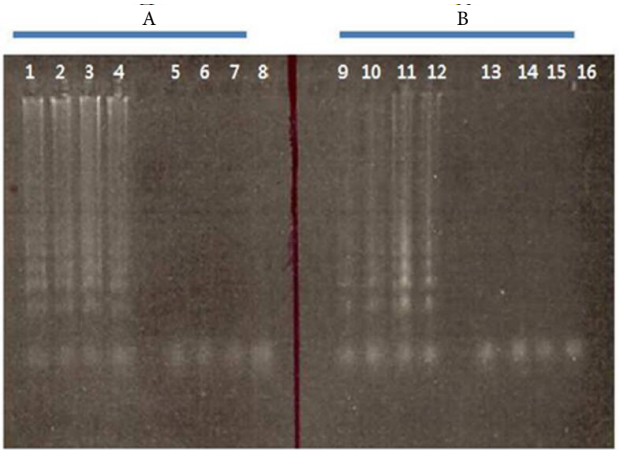

Figure 1. Detection of isothermal amplification products by 1.5% agarose gel electrophoresis. The products were amplified with set 1 (A) and set 2 (B) isothermal amplification primers. Lanes 1 to 4 and 9 to 12 are from female DNA, Lanes 5 to 7 and 13 to 15 are from male DNA, and Lanes 8 and 16 are female DNA samples as a negative control (without primer).

isothermal primers; this was confirmed by the negative control results (Figure 1A, Lane 8) and set 2 (Figure 1B, Lane 16). Therefore, it should be noted that two sets of LAMP primers amplified the sequence of the female target gene of interest.

After these attempts, to demonstrate the simplicity, accuracy, efficiency, and specificity of the these results, isothermally amplified products were examined by direct visualization of the reaction tubes under UV light after adding RedSafe fluorescence dye. The results for this visualization followed a pattern similar to that shown by the results of gel electrophoresis (Figure 2). It was illustrated that samples 1 to 4 and 9 to 12 showed significant amplification by both sets of primers, indicating that those samples corresponded to female birds (Figures 2A and 2B). Moreover, no amplification was observed from samples 5 to 7 and 13 to 15 via UV visualization, clearly indicating that those samples corresponded to the male birds (Figures 2A and 2B). Male samples showed slight bands of artifacts similar to the negative controls (Figure 2, Lanes 8 and 16).

To confirm the structure, the amplified products were digested with two endonucleases; the sizes of the resulting products were then analyzed by electrophoresis. *Hha* I for set 1 LAMP primers cuts between F1c and F2c, and *Bsa*J I for set 2 cuts F1c from the repeat sequence of LAMP products (Figure 3). Consequently, if the amplification products had exactly the same structure, the products would be fragmented to 49-, 171-, 231-, 245-, and 259-bp fragments by Hha I digestion and 39-, 76-, 125-, 106-, 176-, and 192-bp fragments by *BsaJ* I digestion, in good

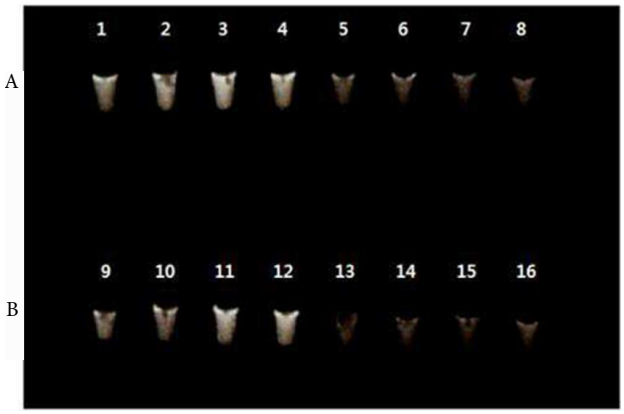

Figure 2. Detection of isothermal amplification products using RedSafe fluorescence dye by direct observation method under a UV light lamp. The products were amplified with the set 1 (A) and set 2 (B) isothermal amplification primers. Lanes 1 to 4 and 9 to 12 are from female DNA, Lanes 5 to 7 and 13 to 15 from are male DNA, and Lanes 8 and 16 are female DNA samples as a negative control (without primer).

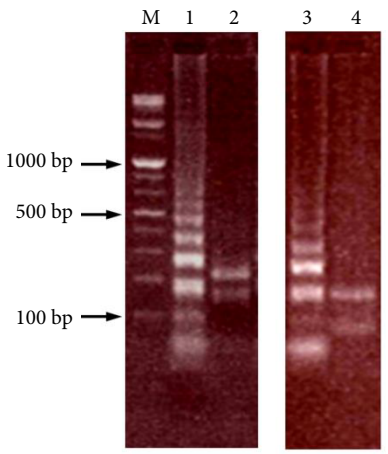

Figure 3. Restriction analysis of the amplified DNA from female genome. Electrophoretic analysis of the LAMP products that were digested by *Hha* I for set 1 primer and *Bsa*J I for set 2 primer, followed by RedSafe staining. Lane M, 100-bp ladder used as a size marker; Lanes 1 and 3, complete LAMP products; Lanes 2 and 4, digested LAMP products.

agreement with the predicted sizes. However, the major bands of 171- and 231-bp fragments were observed from Lane 2 and those of 176- and 76-bp fragments from Lane 4. The intact amplicons were also shown in Lanes 1 and 3.

In addition, it is quite interesting that the sex-specific gene could be detected within 1 h.

4. Discussion

Chicken sexing is an integral part of the breeder, broiler, and layer industries. Early determination of a chick's sex will allow a profitable business. The most important reasons for sexing in the poultry industry are to differentiate the growth rates exhibited by each sex and to separate the egg-producing female birds (15). The poultry industry is in shambles due to the high cost of feed. Therefore, earlier detection will reduce unnecessary costs involved in raising unwanted chickens, such as food, water, and vaccinations. With current methods, sex has to be identified on the basis of morphological observations, examination of sex chromosomes, examination of the gonads by laparotomy or laparoscopy, and behavioral observations (13). However, the above methods have less significance in distinguishing between the two sexes in earlier life stages; they are laborious and time-consuming, with the risk of wrong selection. The early detection of chick sex in the ova in the hatchery would facilitate removal of unwanted chicks and diminish welfare objections regarding culling after hatching (16).

Nowadays, the involvement of molecular techniques in chicken sexing constitutes a breakthrough in the reliability and rapidity of sex identification with more effective ways. The PCR specification method is applied by using the blood or feathers as materials for template DNA (17). However, the success rate of the DNA specification experiment through PCR depends on factors such as sophisticated equipment and availability of skilled hands for proper operation and analysis of the results. Indeed, it is quite difficult and exorbitant for small-scale poultry industry to invest more money. Thus, a more reliable, rapid, rigorous, and reasonable method needs to be promoted.

In this experiment, we demonstrated the efficiency of a newly developed technique (LAMP) for sex determination at an early stage of chicken development. The development of a sexual phenotype generally occurs during embryonic development and is regulated by genetic and hormonal pathways (18). The method in this study was based on the identification of the female-specific gene *PKCIW* from blood DNA, located on the W chromosome, which is involved in female sex determination (19). The study involved the isolation of genomic DNA from the blood samples of two different chicken breeds to analyze the detection of the female-specific *PKCIW* gene. O'Neill et al. (20) reported that *PKCIW* expression has been found in the female genital ridge from day 4 to day 5 in chicken embryos, i.e. prior to sexual differentiation.

We applied the LAMP approach using isothermal amplification primers to analyze the *PKCIW* gene, exhibiting sexual dimorphism in the male and female

chicken embryos. Previous studies also had a similar methodology for the design of isothermal amplification primers (21,22). The mechanism of the LAMP reaction can be explained in three steps: an initial step, a cycling amplification step, and an elongation step (14). Turkyilmaz et al. (23) reported a multiplex PCR protocol for sexing of newly hatched Denizli chicks. Although the technique has shown the permissive steps to identify the sex, it is quite difficult to carry out in a nonlaboratory setting. Moreover, the sexing of the Japanese quail through PCR was reported. The DNA isolated from both embryo and chorioallantoic membranes of Japanese quails was used for identifying the sex of Japanese quail embryos (24). Thus far, the earliest embryos analyzed for sexual differentiation in chickens by this approach have not been determined. LAMP is cost-effective as it does not require special reagents or sophisticated equipment and the assay takes less time for amplification and detection. LAMP provides sequence-specific amplification using only a strand-displacing DNA polymerase. In addition, LAMP uses four core primers recognizing multiple target sequence regions, which confer a high degree of specificity to the reaction (25). These isothermal amplification primers enable the amplification of a small quantity of nucleic acids in a short time without a thermocycling apparatus (26).

Our experiment used two methods for the visualization of amplicons, including gel electrophoresis and direct visualization by using RedSafe dye under UV light. Other studies have also used gel electrophoresis and other fluorescence dyes, e.g., SYBR green methods for the visualization of amplified products (21).

Using the LAMP assay system, we have reported a novel, rapid, and cost-effective detection method for chicken sexing. It was demonstrated that the sex determination in chickens could be possible with short duration times and significant accuracy. Since the discovery of LAMP by Notomi et al. in 2000 (27), LAMP has attracted a lot of attention as a potentially rapid, accurate, and cost-effective nucleic acid amplification method. Currently, these are laboratory results, but we anticipate that further development will allow this method to be used in nonlaboratory settings where PCR cannot be employed.

In conclusion, chicken sex determination is an integral part of the poultry industry. Although the molecular technique in chicken sexing constitutes a breakthrough, certain points in terms of costs and prolonged experimental times can create a barrier for regular application. In the present investigation, we suggest a, simple, easy, and cost-effective method for chicken sexing within the shortest time period. Therefore, the proposed method will be suitable for application beyond the laboratory setting. Moreover, this method can be useful for studies and conservation programs of rare birds. Furthermore, the primers used for sex identification can be used for some other avian species.

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