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A DISSERTATION FOR THE DEGREE OF MASTER OF SCIENCE

Meat species identification using loop-mediated isothermal amplification assay targeting species-specific mitochondrial DNA

종 특이적 미토콘드리아 서열을 활용한 신속유전자등온증폭기법의 육종 감별 적용

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Meat species identification using loop-mediated isothermal amplification assay targeting species-specific mitochondrial DNA

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ABSTRACT

Meat species identification using loop-mediated isothermal amplification assay targeting species-specific mitochondrial DNA

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Meat fraud and adulteration has led to consumer demands for accurate methods of meat source. LAMP assays targeting species-specific mitochondrial DNA were designed to identify and discriminate eight meat species based on annealing curve analysis: cattle, pig, horse, goat, sheep, chicken, duck, and turkey. The LAMP primer sets were designed on the basis of a consensus of several mitochondrial sequences for each species. No cross-reaction was observed and all eight species were successfully discriminated by their unique annealing temperatures. The limits of detection

(LoDs) of the LAMP assays in raw and cooked meat were determined at 10 pg/μl to 100 fg/μl levels, and those in raw and cooked meat admixtures were determined at 0.01% to 0.0001% of target meat levels within 30 min. The newly designed LAMP assays are a simple, rapid, accurate, and sensitive method for discrimination of eight meat species and could be used for meat species identification.

Keywords *:LAMP, meat identification, mitochondrial DNA, meat adulteration, food safety*

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LIST OF ABBREVIATION

BIP	Backward inner primer
BSE	Cattle, pig and horse
B3	Backward outer primer
CO	Goat and sheep
COI and II	Cytochrome oxidase subunits I and II
Cytochrome *b*	Cyt *b*
D-loop	Displacement loop
ELISA	Enzyme-linked immunosorbent assays
FIP	Forward inner primer
F3	Forward outer primer
GAM	Chicken, duck and turkey
LAMP	Loop-mediated isothermal amplification
LB	Backward loop primer
LF	Forward loop primer
LoD	Limit of detection
MtDNA	Mitochondrial DNA
ND2	NADH dehydrogenase subunit 2
ND5	NADH dehydrogenase subunit 5
RAPD	Random amplification of polymorphic DNA

RFLP	Restriction fragment length polymorphism
R^2	Correlation coefficient determinants
SSCP	Single strand conformational polymorphism

INTRODUCTION

As consumption of animal resources increases and varies, excessive and unfair competition between producers has caused problems for consumers of meat-based foods. Several issues include contamination of originally high-valued meat with cheaper substitutes(beef contaminated with horse meat), choices not to intake meat or meat sources due to religious reasons, health problems (allergies) and individual preference (vegetarian) (1, 2). These have led to increased consumer demands for accurate meat identification to combat meat sources fraud and adulteration. In response to these pressures, the industry has developed a wide variety of species identification methods.

Classical methods for meat identification rely on protein analyses, such as immunological, electrophoretic, and chromatographic assays (1, 3, 4). Enzyme-linked immunosorbent assays (ELISA) are used for meat identification by the United States Department of Agriculture Food Safety And Inspection Service (USDA-FSIS). Several researches have reported that ELISA provides accurate meat species identification (5-7). However, protein

analysis has limitations. Protein is not favorable for discriminating processed products such as meat patties or cured, dried, and seasoned meat, because proteins are easily denatured by heat, salt, and pressure (8). Proteins are also unsuitable target for identifying species that are phylogenetically close, such as poultry, because of potential cross-reactivity (9, 10).

Nucleotide amplification methods have been developed as an alternative to protein-based methods. Mitochondrial DNA (mtDNA) has been widely used for species identification, as it is relatively undamaged by food processing and can thus be extracted intact from cooked and processed meats and meat products (9). MtDNA sequences are frequently used instead of nuclear DNA for species-specific identification for several reasons. MtDNA exists in multiple copies (approximately 1,000 copies) per cell (11), is relatively tolerant of environmental conditions such as heat, salt, and pressure. In addition, its rate of evolution facilitates the discrimination of closely related species (12). Several mtDNA-targeting techniques have been described for meat identification, including PCR (11, 13-15), real-time PCR (2, 9, 16-18), PCR-restriction fragment length polymorphism (PCR-RFLP) (19-22), random amplification of polymorphic DNA (RAPD) (23, 24), DNA hybridization (10),

single strand conformational polymorphism (SSCP), and sequencing (25). However, some methods, such as sequencing and DNA hybridization, are expensive and some methods, such as PCR, RAPD, and PCR-RFLP, are generally less sensitive and laborious.

Loop-mediated isothermal amplification (LAMP) is a novel nucleotide amplification method (26). It is specific, rapid, and sensitive because it employs 4 to 6 primers and special DNA polymerases, such as *Bst* or *Gsp* DNA polymerase, which are capable of inducing auto-cycling strand displacement. The reaction occurs under isothermal conditions, eliminating the need for thermal cycling. To our knowledge, the only LAMP assay for species identification has been described with an electronic DNA sensor(27); none have been developed for use with intercalating dye-based analysis systems.

In this study, a real-time LAMP assay was developed for rapid, sensitive, and accurate species identification targeting mtDNA of eight meats: cattle (*Bos taurus*), pig (*Sus scrofa domesticus*), horse (*Equus caballus*), goat (*Capra hircus*), sheep (*Ovis aries*), chicken (*Gallus gallus*), duck (*Anas platyrhynchos*)

and turkey (*Meleagris gallopavo*). Species discrimination was performed based on annealing curve analysis of the LAMP assays. The capability of LAMP assay was assessed by determining the limit of detection (LoD) and by calculating a quantification equation in four sample types: raw meat, cooked meat, raw meat admixtures, and cooked meat admixtures.

MATERIALS AND METHODS

1. Sample preparation

Samples representing eight meat species were purchased from a grocery market in South Korea except horse meat. Horse meat was obtained from the department of veterinary clinical pathology of Seoul National University. Samples (20 mg) were removed for DNA extraction. The admixtures of horse-cattle, sheep-goat, chicken-duck, and chicken-turkey meat were prepared by combining 2 g of horse, sheep, and chicken meat at 0.00001 to 10% in 1.8 g of cattle, goat, duck, and turkey meat. Each sample was then homogenized by chopping with a stainless blade (Dorco, Korea) for 5 min. Heat-treated (cooked) meat samples (20 mg each) were prepared from raw and admixture samples by boiling at 100℃ for 5 min. DNA was isolated with the DNeasy Tissue kit (Qiagen, USA) according to the manufacturer ' s instruction. Briefly, meat (20 mg) was transferred to an empty 1.5 ml micro tube. Buffer ATL (180 μl) and proteinase K (20 μl) were added and mixed by vortexing. The tube was mixed thoroughly by vortexing and incubated at 56℃ until the sample is completely lysed. Buffer AL (200 μl) and ethanol

(200 μl) were added and mixed by vortexing. The mixture was transferred into the DNeasy Mini spin column placed in a 2 ml collection tube. The column was then centrifuged at 8000 rpm for 1 min and the collection tube was discarded. Buffer AW1 (500 μl) was added into the column placed in a new 2 ml collection tube, and the tube was centrifuged at 8000 rpm for 1 min. Buffer AW2 (500 μl) was then added into the column placed in a new 2 ml collection tube, and the tube was centrifuged at 14000 rpm for 3 min. The column was placed in a clean 1.5 ml micro tube and Buffer AE (200 μl) was added directly onto the column membrane. The column was incubated at room temperature for 1 min and then centrifuged at 8000 rpm for 1 min. The DNA sample was then stored at 4℃ or directly used as template.

2. LAMP primer design

To identify each species-specific target, several mtDNA sequences for each species were collected from GenBank. Multiple sequence alignments using CLUSTALW method (Lasergene® 10.1.1, USA) were carried out to develop a consensus sequence. Three sets of primers targeting eight different regions of the consensus sequence were designed by using LAMP designer (Optigene,

UK) before the *in silico* BLAST test provided in the program. The parameters to design the LAMP primers were as below: GC %, 40 to 65; hairpin maximum △G (3’ end), 3.0 –kcal/mol; hairpin maximum △G (internal), 5.0 –kcal/mol; 3’ end maximum △G, 4.0 –kcal/mol; self-dimer maximum △G (3’ end), 2.5 –kcal/mol; self-dimer maximum △G (internal), 8.0 –kcal/mol; run/repeat (dinucleotide) maximum length, 5 bp; G/C clamp – consecutive G/Cs at 3’ end, 1; maximum primer set Tm mismatch, 3.0℃. The LAMP primers are listed in Table 1.

3. LAMP reaction

The LAMP assay reaction conditions using *Gsp*SSD DNA polymerase (Optigene Ltd., UK) were optimized according to the manufacturer’s instructions. Briefly, LoD tests were performed with 0.8 μM inner primers (FIP and BIP), 0.4 μM loop primers (LF and LB), 0.2 μM outer primers (F3 and B3), 1× buffer, 0.5 mMdNTPs, 4 mM $MgSO_2 \cdot 7H_2O$, 1 M betaine, 0.5× Evagreen, 6 U *Gsp*SSD DNA polymerase, and 4 μL DNA template in a 25 μL reaction volume. For the cattle meat samples, 0.2 μM forward and backward inner primers (FIP and BIP), 0.1 μM forward and backward loop primers (LF

and LB), and 0.05 μM forward and backward outer primers (F3 and B3) were added. For the pig meat samples, 0.27 μM FIP and BIP, 0.13 μM LF and LB, 0.07 μM F3 and B3, 3mM $MgSO_2 \cdot 7H_2O$, and 0.8 M betaine were added. For the specificity test, the LAMP sets were organized into BSE (cattle, pig and horse), CO (goat and sheep), and GAM (chicken, duck and turkey) groups, with equimolar primer amounts. Nucleotide-free distilled water was used as a negative control.

Temperature optimization was performed over a range of 60℃ to 67℃ for 30–60 min by using the block gradient function of the Genie® II platform (OptiGene Ltd., UK) before the annealing curve was generated from 98℃ to 80℃, with a 1℃ decrease every 5 seconds. The best reaction temperature for each species was determined as the point at which the amplification ratio curve was inflected in the shortest time. The results were represented on the amplification graph with fluorescence (K) on the Y-axis and time (T) on the X-axis; positivity was determined at which the amplification ratio curve was inflected in 30 min with the mean species-specific annealing temperature.

4. Detection limit

To determine the LoD of the LAMP and PCR assays for raw and cooked samples of each species, 10-fold serially diluted template DNAs with concentrations ranging from 10 ng/μl to 10 fg/μl were tested in triplicate. In the raw and cooked meat admixtures, target species content from 0.00001% to 10 % (from 2 ng to 2 mg) was tested in triplicate. Previously reported primers and conditions were used for the PCR-based comparators (11). Nucleotide-free distilled water was used as a negative control.

For each set, a standard curve based on the LAMP results was generated by plotting the log of the detection times versus the concentrations of template DNA or target species content. The quantification limits of the assay were evaluated by calculating the correlation coefficient determinants (R^2) and quantification equation.

RESULTS AND DISCUSSION

1. Capability of the LAMP assay

The goal of meat species identification is to determine whether particular animal sources are contaminated with other (even banned) species and to prevent food crisis that could become a threat to public health and the food industry. In this study, a rapid and sensitive LAMP assay with intercalating dye detection was developed and eight meat species were successfully identified by annealing curve analysis within 30 min.

MtDNA sequences are often used to discriminate animal species. In this study, we targeted the displacement loop (D-loop), cytochrome *b* (cyt *b*), ATP synthase F0 subunits 6 and 8, and cytochrome oxidase subunits I and II (COI and II) in order to find species-specific non-variable regions and to set the annealing temperatures for successful discrimination (Table 1). These targets have been also used as common markers for nucleotide amplification methods in many research fields. Cytochrome *b* and D-loop genes have

been used for various methods of species identification (2, 9, 13, 15, 19, 28, 29). Meanwhile, several mtDNA genes, such as NADH dehydrogenase subunit 2 and 5 (ND2 and ND5), ATPase 6 and 8, and COI and II, have also used for species identification (17, 18, 20, 30-32).

The optimal reaction temperatures for each LAMP assays were identified as 64.6℃ for cattle and pig, 64.7℃ for horse, 63.5℃ for goat, 64.7℃ for sheep, 65.5℃ for chicken and duck, and 64.6℃ for turkey. It was also demonstrated that the LAMP primers were specific to their corresponding target species. No cross-reactions were observed within the BSE-, CO-, and GAM-LAMP groups. Indeed, no cross-reaction was observed when the single-plex LAMP assays were performed for each of the 3 groups, indicating that the selected markers are suitable for species discrimination (Figure 1-3).

2. Annealing temperature differences

The annealing temperatures differed for each species within the margin of error in the three groups: 85.56℃±0.07℃ for cattle, 84.96℃±0.08℃ for pig, and 85.99℃±0.05℃ for horse in the BSE-LAMP set, 84.91℃±0.11℃ for goat and 83.90℃±0.11℃ for sheep in the CO-LAMP set, and 86.31℃±0.23℃

for chicken, 88.66℃±0.12℃ for duck, and 84.49℃±0.08℃ for turkey in the GAM-LAMP set (Figure 1-3). The annealing curve showed no overlaid peaks between species in each of the sets, revealing that annealing temperature analysis is an excellent tool for LAMP discrimination of these eight raw meat species. The annealing temperature is effective for species discrimination because it differed with the nucleotide composition and amplicon length of each target (33, 34). In contrast to probe-based real-time PCR techniques, intercalating dye-based methods have many advantages. Target detection with specific probes is more expensive than using general intercalating dyes. In addition, it is difficult to design and optimize a probe (35). For these reasons, melting curve analysis after SYBR green-based real-time PCR has been used to identify ruminant (cattle) and poultry (turkey) in foodstuffs (36), discrimination of plum pox virus isolates of strain D and M (35), and discrimination of deer and six common domestic species (37).

3. LAMP assay LoDs in raw and cooked meat samples

The LoDs of LAMP and PCR assays in raw and cooked meat samples are presented in Table 2. Amplification curves corresponding to samples at 10

ng/μl to 1 pg/μl of target DNA were generated for pig, horse, goat, sheep, and turkey raw meat; samples of 10 ng/μl to 10 pg/μl of target DNA were generated for cattle, chicken, and duck meat. Amplification curves corresponding to samples at 10 ng/μl to 100 fg/μl of target DNA were generated for cooked chicken, and from 10 ng/μl to 1 pg/μl of target DNA for the remaining cooked samples, indicating that the LAMP assays are more sensitive than PCR by 10^2 to 10^3 times and 10 to 10^4 times in the raw and cooked meat samples, respectively, with strong linear relationships between detection time and template mtDNA concentration. Studies of raw horse and donkey meat samples by TaqMan-based real-time PCR have yielded LoDs of 1 pg DNA in water (38); the LoD for raw cattle meat is 35 pg DNA (39). A study that employed a LAMP method and electrochemical DNA sensing described LoDs for pork, bovine, and chicken samples as 20.33, 23.63, and 78.68 pg/μl (27), indicating that the LAMP assays were more sensitive in these species.

Improvement of the R^2 value of cooked duck and turkey samples was observed at 10 pg/μl, 10-fold greater than the LoD (0.78 to 0.99 and 0.79 to 0.96 in duck and turkey, respectively). It indicates that the LAMP assays

may be further assessed as one of the oligo-nucleotide quantification tools, such as real-time PCR. In comparison to the raw meat samples, LoDs were the same or lower in cooked meat samples. Indeed, where the same LoDs were found, the detection times were faster in cooked samples than in raw ones with the exception of goat, sheep, and turkey. Target regions under 150 bp are not influenced by heat treatment (17); thus, the amplified regions in our study should not be affected by cooking. Since the optimal size of the amplified region in LAMP is below 200 bp (40), the method may be used for even highly degraded DNA targets.

Cattle and pork are the most favored meat sources in South Korea. In Europe, large quantities of horse meat are produced and imported for human consumption, as cattle meat is relatively expensive (41). The high cost of cattle meat could lead to intentional contamination with pig and horse meat. In goat and sheep, the authentication of origin is important because of the close relationship between these ruminant species. In dairy industry, the substitution of origin sources with other inferior product could be issued, because sheep and goat sources have more nutrients and economic advantages in comparison to cow-derived products (42) and sheep

sources are more costly than goat sources worldwide (43). In addition, to our knowledge, no other studies have aimed to discriminate goat and sheep. Therefore, the LAMP method could facilitate fast and sensitive discrimination of these small ruminant species. Poultry, chicken, duck, and turkey have been also adulterated for economic reasons. Foie gras from goose and duck have been contaminated with less expensive sources such as chicken or turkey (44). As the consumers who intake foie gras and other products have been increased nowadays, high quality and authentic foods provided to consumers is absolutely challenging due to such adulteration cases. Our LAMP assays could be used for fast and sensitive monitoring for food fraud.

4. LAMP assay LoDs in raw and cooked meat admixtures

In the admixture samples, horse, sheep, and chicken meat were targeted because they are considered cheap sources of adulteration. The amplification curves of each target species were generated based on detection time (T_t) and fluorescence for10 to 0.0001% (2 mg to 2 ng) target in non-target species admixtures within 30 min. The LoDs of LAMP and PCR assays in raw and cooked admixture samples are presented in Table 3. Amplification

curves for samples of 10 to 0.01% were generated for the chicken-duck raw meat admixture; samples of 10 to 0.001% were used to generate curves for the remaining raw meat admixture samples. Samples of 10 to 0.001% were used to generate curves for sheep-goat and chicken-duck cooked meat admixtures, and samples of 10 to 0.01% and 10 to 0.0001% were used to generate curves for cooked horse-cattle and chicken-turkey admixtures, respectively. Thus, the LAMP assays were more sensitive than PCR by 10^2 to 10^4 fold. The LoD of PCR in heated ovine samples mixed with oats is 0.1% (45); in poultry and ruminant species, samples with vegetable contamination (maize) were reported at 0.002% (46). However, these results cannot be directly compared because an LoD expressed as meat content (w/w) does not show the absolute capability of the assay (47). Further study may be needed for the correct evaluation of studies with a consensus unit, such as DNA/DNA equivalents (47).

The annealing temperature of LAMP amplicons was fell within the unique range of each species and negative control was not amplified in all of the experiments. Compared to the raw meat samples, no difference was observed in LoD for the sheep-goat raw and cooked admixture samples; the

LoDs for chicken-turkey and chicken-duck cooked admixtures were lower than that of the raw admixtures. Detection times were shorter for cooked admixtures than for raw ones (Table 3), perhaps because heating may influence the extraction efficiency of mtDNA from the sheep-goat, chicken-turkey, and chicken-duck samples. In contrast, the LoDs in cooked horse-cattle admixtures were greater than in the raw admixtures; detection times and detection limits were higher for chicken-duck than for chicken-turkey admixtures. It may be attributable to DNA from the non-target meat could inhibit target DNA amplification (9), although the annealing temperature in raw and cooked horse-cattle admixtures was 85.75℃ ± 0.16℃ and 85.59℃ ± 0.22℃, respectively, indicating successful amplification of horse mtDNA. Since the LAMP method is generally very robust against pH, temperature changes, and even reagent exposure, it is considered to be more sensitive and specific than other nucleotide amplification methods (48). The LAMP assays could be used as tools for discrimination and quantification of meat species in unknown and heat-treated samples.

The LAMP assays combined with annealing curve analysis were developed to identify and discriminate eight meat species. The LAMP primers were

designed for specific amplification of mtDNA regions in each target species. The annealing temperatures for each species were unique in three sets. No cross-reaction was observed in cattle-pig-horse (BSE), goat-sheep (CO), and chicken-duck-turkey (GAM) samples. The detection limits of the LAMP assays in raw meat, cooked meat, raw admixtures, and cooked admixtures were determined in 30 min and revealed greater sensitivity than PCR assays. The LAMP assays are simple, rapid, accurate, and sensitive for discrimination of eight meat species and could be used to support meat species identification for eradication of meat source fraud and adulteration. Further study may be needed to verify the utility of the LAMP assays for other animal products such as milk, milk powder, cheese, and smoked or frozen meats.

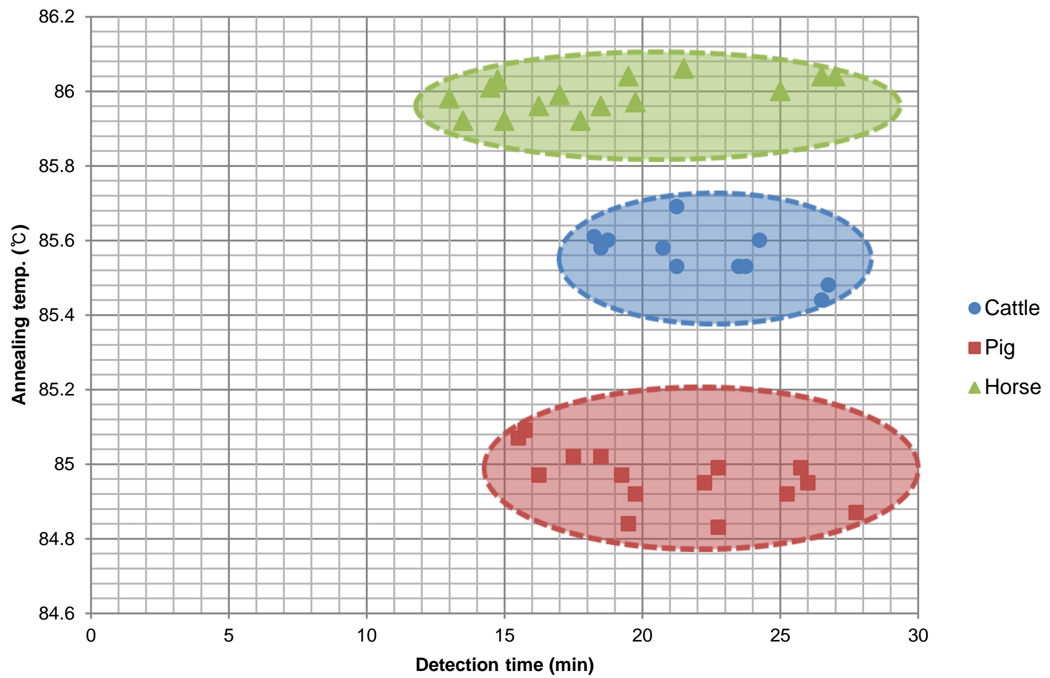

Figure 1. The annealing temperature and detection time graph of cattle, pig and horse meat. The results were presented on the graph with annealing temperature (℃) on the Y-axis and detection time (min) on the X-axis.

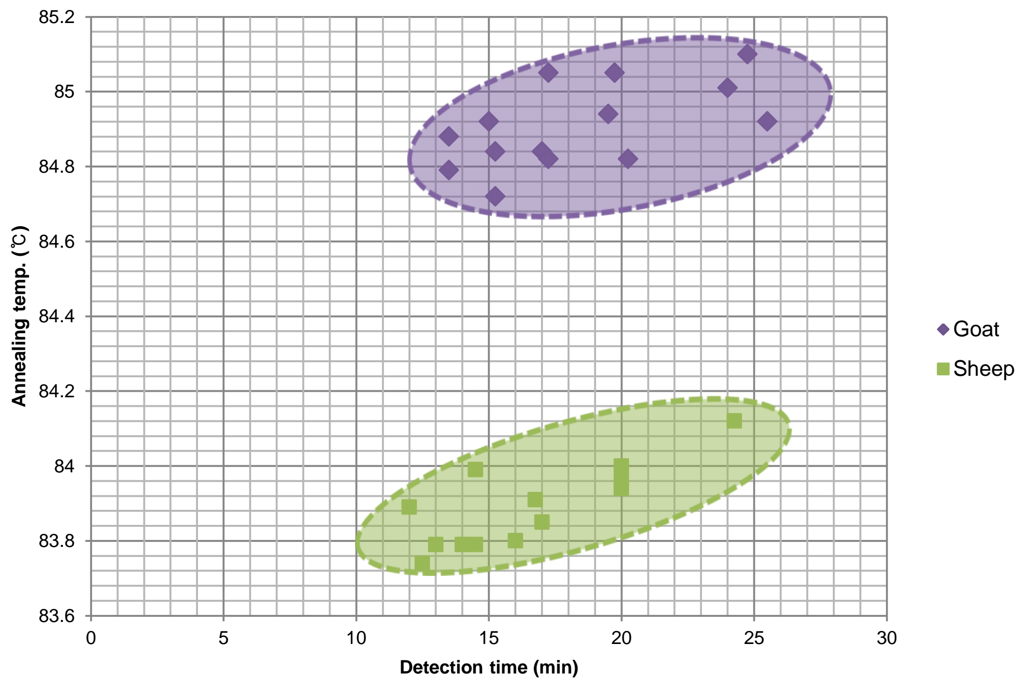

Figure 2. The annealing temperature and detection time graph of goat and sheep meat. The results were presented on the graph with annealing temperature (℃) on the Y-axis and detection time (min) on the X -axis.

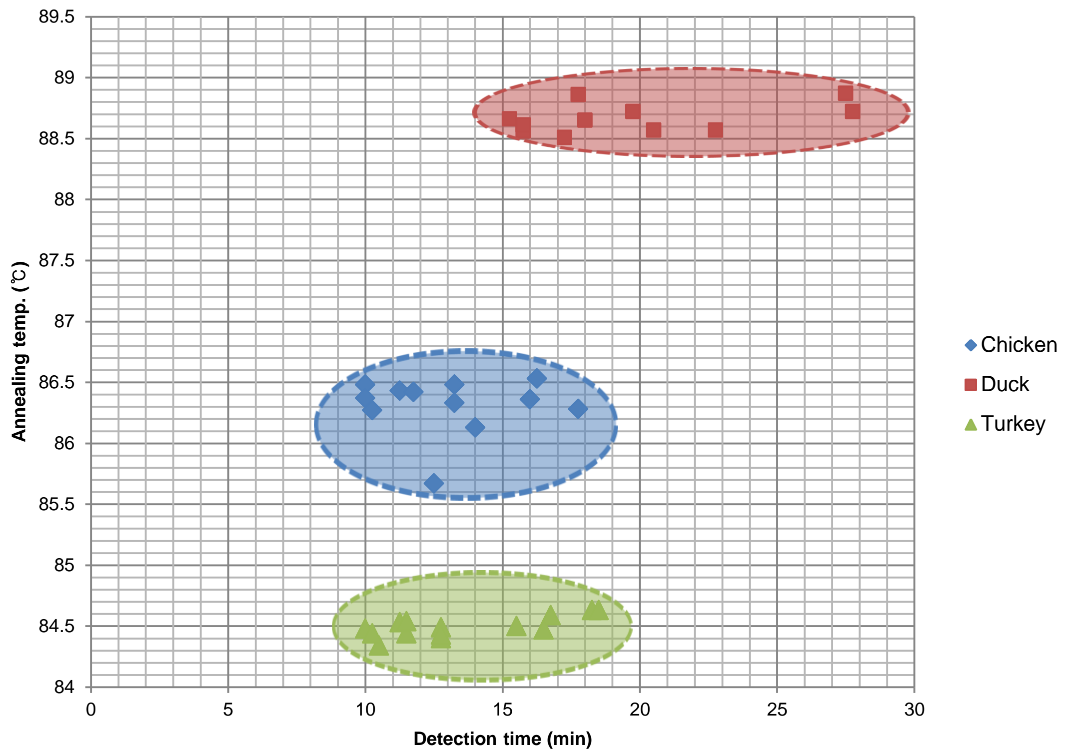

Figure 3. The annealing temperature and detection time graph of chicken, duck and, turkey meat. The results were presented on the graph with annealing temperature (℃) on the Y-axis and detection time (min) on the X-axis.

Table 1. LAMP primer sequences and their target regions for 8 meat species

Scientific name			Sequences (5' to 3')	Accession No.	Gene
Bos taurus	Cattle	FIP	TCCAGCTACAATAGATGCTCCGACACATAACTGTGCTGTCAT	AY526085	D-loop
		BIP	GCATCTTGAGCACCAGCATAAAGTGGTGGTAGATATTTAAGGG	AY676864	
		LF	ATAGCTGAGTCCAAGCATCC	DQ124417	
		LB	CAGTCAATGGTCACAGGACA	JN817306	
		F3	GCTAATCAGCCCATGCTC	NC006853	
		B3	TTGACTTTGTTTGGAGTGCT		
Sus scrofa domestica	Pig	FIP	AGGGATGGGACGGCTCATGACAATCGAGTTGTTCTACCA	KC469586	cytochrome oxidase subunit II
		BIP	ACAGATGCTATCCCAGGACGATCTGAGCACTGTCCGTAA	KC469587	
		LF	GCAGTACGTCTTCAGAGGATAC	NC012095	
		LB	CTCTAATATCCACACGACCTGG	AP003428	
		F3	AGACTATGAAGACCTCACCTT		
		B3	AGTGCTGACTAGCTTCTCA		
Equus caballus	Horse	FIP	CGTGGGTTTGGTGGGTCATTATTCGCAGTAGCTATAATCCAAG	AY584828	ATP synthase F0 subunit 6
		BIP	CCCAGCCCATGACCACTTACACATGGCTAGTCCTGATGT	EF597513	
		LF	GGCTTACCAGGAGAGTGAATAC	EU939445	
		LB	CCCTATCAGCCCTCCTGA	FJ718997	
		F3	ACACCTCCTAATACACCTCAT	NC001640	

		B3	TCGGATGATGTCTCGTCA		
Capra hircus	Goat	FIP	TGTTGGCGTTTGTGTGGGTATACAGACATGCCAACAACC	GU068049	D-loop
		BIP	CACACAATGTTACGCGTATGCAGTCCGCGTTATATGGATGTTA	GU229280	
		LF	TTGGGTTAGGATTGGGATGTTT	GU229281	
		LB	AGTACATTACACCGCTCGC	GU295658	
		F3	AACACAAACTTCCCACTCC	NC005044	
		B3	GCTGGATTAGTACTGCATATGT		
Ovis aries	Sheep	FIP	GGGAGTGTTAAGTGGGTTTGCTTAGTACTATTCACGCCTGACT	HM236179	cytochrome b
		BIP	CCATACATCAAAGCAACGGAGCAGGTCGGCTACTAGGATTC	HM236175	
		LF	TTGTCTGGGTCTCCGAGT	HM236183	
		LB	TTCCGACCAATCAGTCAATGT	NC001941	
		F3	CTACTAATCCTCATCCTCATGC	HM236185	
		B3	CCTCCAATTCATGTGAGTGT	AY858379	
Gallus gallus	Chicken	FIP	GGCTGGAAGAAGGAGTGATGGCCTGAACCTGACCATGAAC	AP003323	ATP synthase F0 subunit 8 ATP synthase F0 subunit 6
		BIP	ACTTCCATCACCAGGAAACCGATCAGAGTTGGATGGTGGA	AP003580	
		LF	CTTGAGAATTGGTCGAAGAAGC	AY235570	
		LB	TGGATCAACAACCGCCTC	AY235571	
		F3	CCATGATTCTCCATCATACTCC	NC001323	
		B3	AGGATAAGTGAGGTGAGTAGG		
Anas platyrhynchos	Duck	FIP	GGTTGCCTGCTAGAGGTGGACCATCATTCCTCCTTCTACT	EU009397	cytochrome oxidase subunit I
		BIP	CCTCAGTGGACCTGGCTATCTGAAGTTAATGGCTCCGAGG	EU755252	

		LF	CCTCATCCACTGTAGAAGCTG	EU755253	
		LB	CTCACTTCACCTGGCTGG	FJ167857	
		F3	TTCGGCAACTGATTGGTC	NC009684	
		B3	CATCCTGCTCCTCCTATCA		
Meleagris gallopavo	Turkey	FIP	AATCGTCCTGGGATTGCATCTGGCTGACGATGTATTACACTCA	EF153719	
		BIP	ACCAGGAGTGTTCTACGGACAGGATTCTACTACGATAGGCATG	JF275060	
		LF	ACTCCGAGGGTTGGTACA	NC010195	D-loop
		LB	GCGGAGCTAACCACAGTT		
		F3	AGTTGACCACCGTATAGTAGT		
		B3	AGGCTAGTGCTGATTCCA		

Table 2. Detection time and limit of raw and cooked meat samples of 8 species

Detection time (min) / Template concentration	Cattle				Pig				Horse				Goat			
	Raw		Cooked		Raw		Cooked		Raw		Cooked		Raw		Cooked	
	LAMP	PCR	LAMP	PCR	LAMP	PCR	LAMP	PCR	LAMP	PCR	LAMP	PCR	LAMP	PCR	LAMP	PCR
10 ng	18.5±0.3	+	16.3±0.0	+	15.8±0.4	+	16.5±0.3	+	13.7±0.8	+	11.8±0.0	+	13.5±0.0	+	14.3±0.9	+
1 ng	21.1±0.3	+	19.1±0.1	+	18.4±0.9	+	18.8±0.4		15.3±0.8	+	13.0±0.3		15.2±0.1	+	16.0±1.1	+
100 pg	23.8±0.4	+	22.0±0.3	+	20.5±1.5	+	20.7±0.1		17.8±0.8	+	15.2±0.4		17.2±0.1	+	18.1±1.4	+
10 pg	26.6±0.2	+	24.8±0.6	+	23.8±1.9	+	22.8±1.0		20.3±1.1		16.5±0.0		19.8±0.4		21.0±1.6	+
1 pg					26.3±1.3	+	25.9±0.5		26.2±1.0		18.5*		24.8±0.8		24.7±3.2	
100 fg																
10 fg																
R^2	0.9930		0.9933		0.9204		0.9717		0.9279		0.8552		0.9399		0.8407	
T_m(° C)	85.56 ±0.07		85.12 ±0.07		84.96 ±0.08		84.82 ±0.08		85.99 ±0.05		85.74 ±0.09		84.91 ±0.11		84.83 ±0.11	
Detection time (min) / Template concentration	Sheep				Chicken				Duck				Turkey			
	Raw		Cooked		Raw		Cooked		Raw		Cooked		Raw		Cooked	
	LAMP	PCR	LAMP	PCR	LAMP	PCR	LAMP	PCR	LAMP	PCR	LAMP	PCR	LAMP	PCR	LAMP	PCR
10 ng	12.5±0.5	+	13.9±0.3	+	10.1±0.1	+	8.9±0.3	+	15.6±0.3	+	12.3±0.1	+	10.3±0.3	+	10.2±0.3	+

1 ng	14.3±0.3	+	15.8±0.1	+	11.8±0.6	+	10.2±0.3	+	17.7±0.4	+	14.3±0.3	+	11.4±0.1	+	11.2±0.1	+
100 pg	16.6±0.5	+	18.0±0.3		13.5±0.4		11.7±0.1		21.0±1.6	+	16.0±0.0	+	12.8±0.0		12.8±0.0	
10 pg	20.0±0.0		20.5±0.5		16.7±0.9		13.6±0.6		27.6±0.2	+	18.3±0.3		16.3±0.7		14.3±0.7	
1 pg	24.3*		23.0±1.1				14.9±0.6				29.5±0.0		18.4±0.2		21.3±2.2	
100 fg							17.8*									
10 fg																
R^2	0.9599		0.9768		0.9338		0.9683		0.9023		0.7814		0.9446		0.7912	
T_m(° C)	83.90 ±0.11		83.89 ±0.11		86.31 ±0.23		86.40 ±0.10		88.66 ±0.12		88.58 ±0.12		84.49 ±0.08		84.74 ±0.08	

*, One repeat was observed in the triplicate.

Table 3. Detection time and limit of raw and cooked meat admixture samples

Composition of target meat (%) \ Detection time (min)	Horse-cattle				Sheep-goat				Chicken-duck				Chicken-turkey			
	Raw		Cooked		Raw		Cooked		Raw		Cooked		Raw		Cooked	
	LAMP	PCR	LAMP	PCR	LAMP	PCR	LAMP	PCR	LAMP	PCR	LAMP	PCR	LAMP	PCR	LAMP	PCR
10	12.3±0.4	+	12.8±0.0	+	16.7±0.7	+	13.3±0.0	+	10.2±0.5	+	9.3±0.0	+	9.0±0.4	+	9.1±0.1	+
1	13.7±0.3	+	14.1±0.1		17.8±0.8		15.8±0.6	+	11.1±0.1	+	10.0±0.5	+	10.1±0.5	+	10.6±0.1	+
0.1	15.7±0.5		16.6±0.1		19.6±0.8		17.6±0.4		12.8±0.0		11.6±0.4		11.8±0.7	+	12.1±0.1	
0.01	17.7±0.6		22.0*		22.3±0.7		19.8±0.4		15.0±0.8		13.4±0.5		13.6±0.4		14.0±0.3	
0.001	22.3*				29.4±0.2		26.0±3.2				18.8±1.1		16.2±1.9		17.3±1.6	
0.0001															17.5±0.7	
0.00001																
R^2	0.9255		0.8856		0.8430		0.8506		0.9279		0.8552		0.8939		0.9359	
T_m(°C)	85.75±0.16		85.59±0.22		83.96±0.12		83.86±0.19		86.35±0.15		86.27±0.11		86.34±0.11		86.32±0.12	

*, One repeat was observed in the triplicate.

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국문초록

종 특이적 미토콘드리아 서열을 활용한 신속유전자등온증폭기법의 육종 감별 적용

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최근 일련의 식육불법 혼입 사건 등은 이와 같은 식육 불법 혼입을 정확히 판별해 낼 수 있는 과학적인 기법에 대한 소비자의 요구를 증가시켰다. 이러한 소비자의 요구를 충족시키고자 식육 감별을 위한 유전자증폭기법은 다양하게 개발 및 활용되어 왔다. 본 연구에서는 종 특이적 미토콘드리아 서열을 활용한 신속유전자등온증폭기법을 개발하여 소, 돼지, 말, 염소, 양, 닭, 오리, 칠면조 총 8종의 식육을 특이적으로 검출 및 감별하고자 하였다.

종 특이적 미토콘드리아 서열을 활용하여 LAMP 프라이머를 제작하여 각기

다른 annealing temperature가 설정되도록 하였고, LAMP 시약 내 intercalating dye를 첨가하여 반응산물의 증폭 및 분석을 실시간 관찰할 수 있도록 하였다. 각각의 LAMP기법별 최적 반응 온도 및 시약 조건을 설정하였고 8종의 식육을 3그룹으로 분류한 후 시험한 결과, 교차 반응 없이 모두 100%로 관찰되었으며 각 종마다 고유한 값을 나타내어 이를 토대로 그룹 내에서 특정 식육 종을 성공적으로 분류할 수 있었다.

개발된 LAMP기법을 원료육, 가열육, 혼합육, 혼합가열육 등 총 4개의 시료군에 적용하여 검출한계를 관찰하였다. 원료육 및 가열육에서의 LAMP 검출한계는 10 pg/μl to 100 fg/μl의 범위에서 관찰되었고, 혼합육 및 혼합가열육에서의 LAMP 검출한계는 0.01% to 0.0001%의 범위에서 관찰되었다. 또한, 실시간으로 관찰된 증폭산물을 토대로 30분 이내에 정량화 곡선을 도출할 수 있었기에 매우 신속하고 실시간 정량이 가능한 기법임을 알 수 있었다.

본 연구에서 개발한 LAMP기법은 신속성, 민감도, 정확도가 높은 유전자 분석 기법으로서 식육 종의 감별을 보다 쉽게 수행할 수 있을 것으로 기대되며, 향후 표준법과의 병행 활용 가치 또한 높을 것으로 기대된다.

Keywords *:LAMP, meat identification, mitochondrial DNA, meat adulteration, food safety*

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