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Research Article
Visual Loop-mediated Isothermal Amplification (LAMP) Method for Identification Bovine and Ovine Gene in Animal Foodstuff

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Abstract

Objective: The aim of this study was to develop an easy and highly sensitive visual loop-mediated isothermal amplification (LAMP) assay for specific detection of the bovine and ovine derived ingredient in foodstuff. **Methodology:** The primers designed based on different regions of mitochondrial DNA (CoI and Cyt b) for amplification of 184 and 206 bp fragments, respectively. The specificity of assay was cross tested with DNA of horse, pork and chicken, where amplification were observed in bovine and ovine without cross reactivity with others meat species. **Results:** The sensitivity was 5.0×10^{-3} ng for bovine, as same as the ovine genomic DNA. The detection limit of meat mixtures was 0.01% for bovine and 0.001% for ovine. Moreover, the LAMP assay was in concordance with the qPCR analysis in the test of practical samples. The visual LAMP assay can be considered as a valid tool for rapid detection of animal derived materials in meat products. **Conclusion:** It contributes to a future approach on food analysis by meeting the demands for quick and easy-to-perform analytical methods.

Key words: Loop-mediated isothermal amplification, meat species identification, bovine, ovine, foodstuff

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In 2015, the total meat production in China approximated 86.2 million tons of which, 7.0 million was beef and 4.4 million was mutton. Figures released recently by National Bureau of Statistics of the People's Republic of China show that the demand for meat is projected to escalate steadily over the next decade as a result of increasing consumption. With technology advances in the meat processing industry, adulteration and fraud have become common due to monetary benefits of substituting cheaper and more widely available produce[1-4]. Cattle jerky, for example, is used instead of yak jerky as a substitute ingredient. Now-a-days, the identification of meat species and the authentication of its status are major concerns in many countries. For the sake of public health, commercial protection and religious customs, it is imperative to develop an applicable method to authenticate meat origin.

In the past decades, several reliable and powerful detection methods have been developed, including protein-based[5] and DNA-based approaches[6-8]. Different processing conditions, such as the temperature, had a great influence on the test results of enzyme-linked immunosorbent assay (ELISA)[9]. The inspection and quarantine system had developed the industry standard and national standard for cattle and sheep derived materials based on PCR method in China[10] and qPCR method was able to quantitative analyze[11]. However, these methods were operated in fully equipped laboratories with good infrastructure, time consuming and with highly trained staff. Rapid high-throughput methods are needed to facilitate controls to ensure proper labelling in the future and in compliance with the law.

Loop-mediated isothermal amplification (LAMP) was a novel nucleic acid amplification procedure, invented by Notomi[12] in 2000. It had four primers (F3, B3, FIP and BIP) binding six distinct regions and amplified target fragments by Bst DNA polymerase with strand displacement activity at reaction temperature between 60 and 65°C. Because of no need for advanced equipments and expensive reagents, the method had been widely applied in discriminating meat species[13-15]. The use of calcein was convenient for researchers to observe the result by the naked eyes directly[16]. A positive reaction was indicated by a color change from yellow to fluorescent green. Furthermore, it could reduce the risk of pollution generated in amplification process and eliminated the need to open the tubes after reactions.

In this study, we had tried to determine cattle and sheep source in foodstuff by using visual LAMP technique. The method for identification of the animal origin of meat affects the food and meat industry, trade, markets, the restaurant industry and other fields.

MATERIALS AND METHODS

Preparation of meat samples: To make DNA templates for specificity testing, different fresh meat samples were collected from the local municipal slaughter houses, including *Bos taurus*, *Ovis aries*, *Equns caballus*, *Sus scrofa* and *Gallus gallus*, while commercial meat products for testing were obtained in local supermarkets, such as ham sausage, dried beef cubes, mutton rolls and so on. Standard meat products used in the present study were provided by Zhejiang Food and Drug Administration. In addition, a total of 489 blind samples were also provided by Zhejiang Entry-exit Inspection and Quarantine Bureau.

DNA extraction: The meat samples needed to be pre-cooked for 30 min in boiling water for removal of protein as much as possible and then all were grinded into powder with liquid nitrogen in a mortar. Genomic DNA was got from 50 mg of each sample, using the universal genomic DNA extraction kit (TaKaRa), according to the manufacturer's protocol. All extractions were stored at -20°C until be tested. The DNA concentration was measured by a spectrophotometer (DU730, Beckman Coulter, USA).

Primers and probes design: The sequences were obtained from GenBank in NCBI database (accession numbers: Bos Taurus, HM102290.1; Ovis aries, FJ785335.1). After aligning by using Clustal W software, the specific regions were selected as the target fragment. Based upon the detailed analysis and comparison, specific primers of LAMP including loop-primers were designed with the online tool Primer Explorer V3.0 (http://primerexplorer.jp/e/), which was produced by Eiken Chemical (Tokyo, Japan). Meanwhile, primers for a TaqMan-based qPCR were designed by Beacon Designer 7.9, which used FAM and VIC fluorescent dyes to sign. All primers were shown in Table 1, which were synthesized by Invitrogen Biotechnology Co. Ltd.

Real-time PCR protocol: The reaction mixture was added in a total volume of 20 and 2.5 μL of each DNA template was added following the monitor by using the instrumentation (7500 Fast Real-time System, ABI, USA) for qPCR assay. It contained 10 μL of SensiFAST Probe Lo-ROX (Bioline, USA), 0.4 μM of primer sets, 0.1 μM of probe and the rest filled with ddH_2O. The thermal cycle program was described as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 26 sec.

Visual LAMP amplification: Calcein, a metal indicator that yields strong fluorescence by forming complexes with

Table 1: The LAMP primers and real-time PCR primers for *B. taurus* and *O. aries*

Primers	Sequence (5′-3′)	Target gene	Fragment length (bp)
LAMP primers			
Bovine-F3	TCTTTACACTTAGCAGGAGT	*Bos taurus*	184
Bovine-B3	ATAGCATTGTGATGCCGG	Cytochrome oxidase subunit 1	
Bovine-FIP	GCGGGGGGCTTTATGTTGATTTTTTTCCTCAATTTTAGGAGCCATC		
Bovine-BIP	AATGTCACAATACCAAACCCCTCTTTTTAATACAGGGAGCGAGAGT		
Bovine-loop B	TCGTATGATCCGTAATAATTACCGC		
Ovine-F3	GCACAAACCTAGTCGAATG	*Ovis aries*	206
Ovine-B3	GGTGTAATAAGGGTGGAAGG	Cytochrome b	
Ovine-FIP	TGGGAAAATAAAGTGAAAGGCGAAATTTTTGGGGAGGATTCTCAGTAG		
Ovine-BIP	CAGCCCTCGCCATAGTTCACTTTTGTGTCCGATGGAATTCCTG		
Ovine-loop B	AATCGGGTGAGGGTAGCTTT		
Ovine-loop F	TCTTCCTCCACGAAACAGGA		
qPCR primers			
Bovine FP	CCTCTGTTCGTATGATCC	*Bos taurus*	95
Bovine RP	CGGTCTGTTAATAGCATTG	Cytochrome oxidase subunit 1	
Bovine probe	CGCCGTACTACTACTACTCTCGC		
Ovine FP	CGCCTTTCACTTTATTTTC	*Ovis aries*	180
Ovine RP	GAGGATGAGGATTAGTAGG	Cytochrome b	
Ovine probe	CCTCGCCATAGTTCACCTACTCTTC		

divalent metallic ions, such as calcium and magnesium, is used for various analyses[16]. The absorption and emission wavelength of calcein were 497 and 515 nm, which were much closed to the SYBR Green I dye. Quantitative PCR instrument was used for real-time monitoring the LAMP reaction. The time, produced fluorescence, was called the Amplification Start Time (AST). The program was described as follows: 65°C for 1 min, followed by 60 cycles of 65°C for 60 sec. It acquired the signal readings every 1 min. At last, it was terminated by heating at 80°C for 5 min.

Each of the DNA template was added 2.5 μL to the amplification reagent containing LAMP primers together with 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 10% Tween 20 (Sigma-Aldrich), 0.8 M betaine, 0.2 mM calcein, 0.5 mM $MnCl_2$, 1.4 mM dNTPs (Thermo Scientific, USA) and Bst DNA polymerase (8 U μL^{-1}, NEB, USA).

RESULTS AND DISCUSSION

Optimization of visual LAMP assay: The amplification efficiency would be enhanced if we optimized the parameters in the reagents, such as the salt concentrations of magnesium ions, the concentration of calcein and the ratio among outer primers and inner primers. The same conditions were repeated twice times.

The reaction was conducted with different concentration of magnesium ions, which ranged from 0-14 mM. According to the AST, the final concentration of Mg^{2+} was determined 8 mM (Fig. 1a).

The concentration of calcein was set to 200, 300, 400, 500, 600, 700 and 800 μM. To distinguish clearly by the naked eyes, the final calcein concentration was 200 μM (Fig. 1b).

The inner primer of the LAMP reaction was mainly involved in the synthesis of the latter stem-loop structure and the outer primers only participated in the reaction in the initial reaction[12]. Therefore, it had better fix the concentration of the outer primers and the inner primer concentration was changed gradually. The ratios were set to 1:1, 1:2, 1:4, 1:8 and 1:16. The outer and inner primer ratio was finally identified as 1:8 (Fig. 1c).

Sometimes, loop primers would be added to accelerate the reaction[17]. Nevertheless, a set of loop primers for the bovine cut down the amplified efficiency. Finally, only added LB primer into the reaction system. In addition, adding more enzymes could shorten the reaction time effectively but could not improve the sensitivity.

Analytical specificity of visual LAMP assay: In order to verify the specificity of the primers of bovine and ovine, five livestock species: bovine, ovine, horse, pork and chicken were carried out by visual LAMP assay. All samples used in specificity were tested in 3 replicates.

As expected, only the samples represented to the cattle or sheep genomic DNA, had significant changes in fluorescence with real-time instrument. The fluorescent green color of the reactions was obtained as positive. If the color was orange, it meant there were no amplified products in other animals DNA or NTC (No template control) during 60 min (Fig. 2a, b). As a comparison, the real-time PCR assay also showed the same results (Fig. 2c, d). The results indicated the specificity of the detection of *Bos taurus* and *Ovis aries*, respectively.

The high specificity of the visual LAMP assay was provided by the use of three primer sets, all of which had to bind

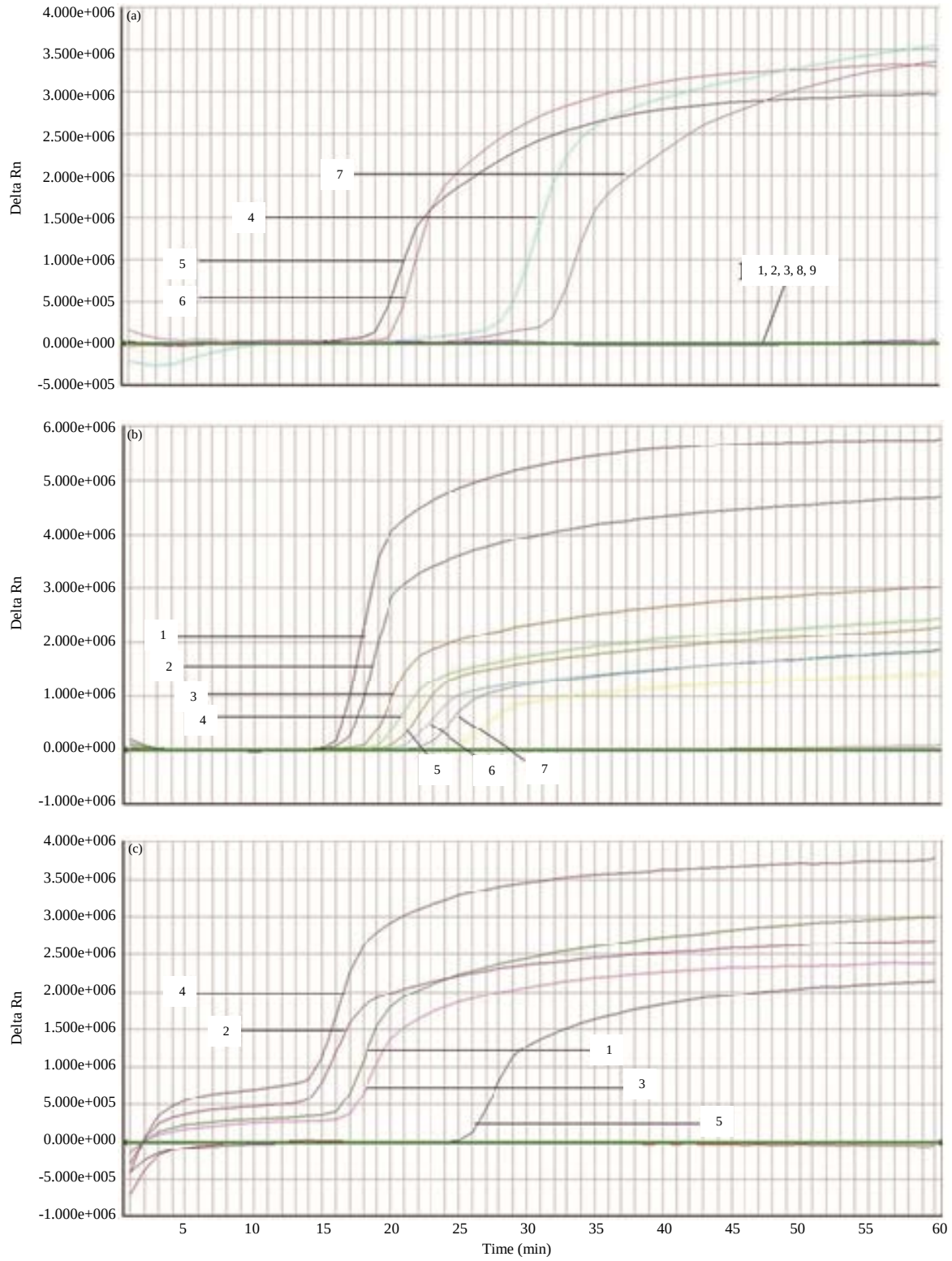

Fig. 1(a-c): Optimization of the LAMP conditions, (a) Different final concentration of Mg^{2+}; 1-8: The final concentration of Mg^{2+} with 0, 2, 4, 6, 8, 10, 12 and 14 mM, 9: No template control, (b) Different final concentration of calcein, 1-7: The final concentration of calcein with 200, 300, 400, 500, 600, 700 and 800 μM and (c) Different ration of the outer and inner primer concentration, 1-5: The ratio of outer and inner primers with 1:1, 1:2, 1:4, 1:8 and 1:16

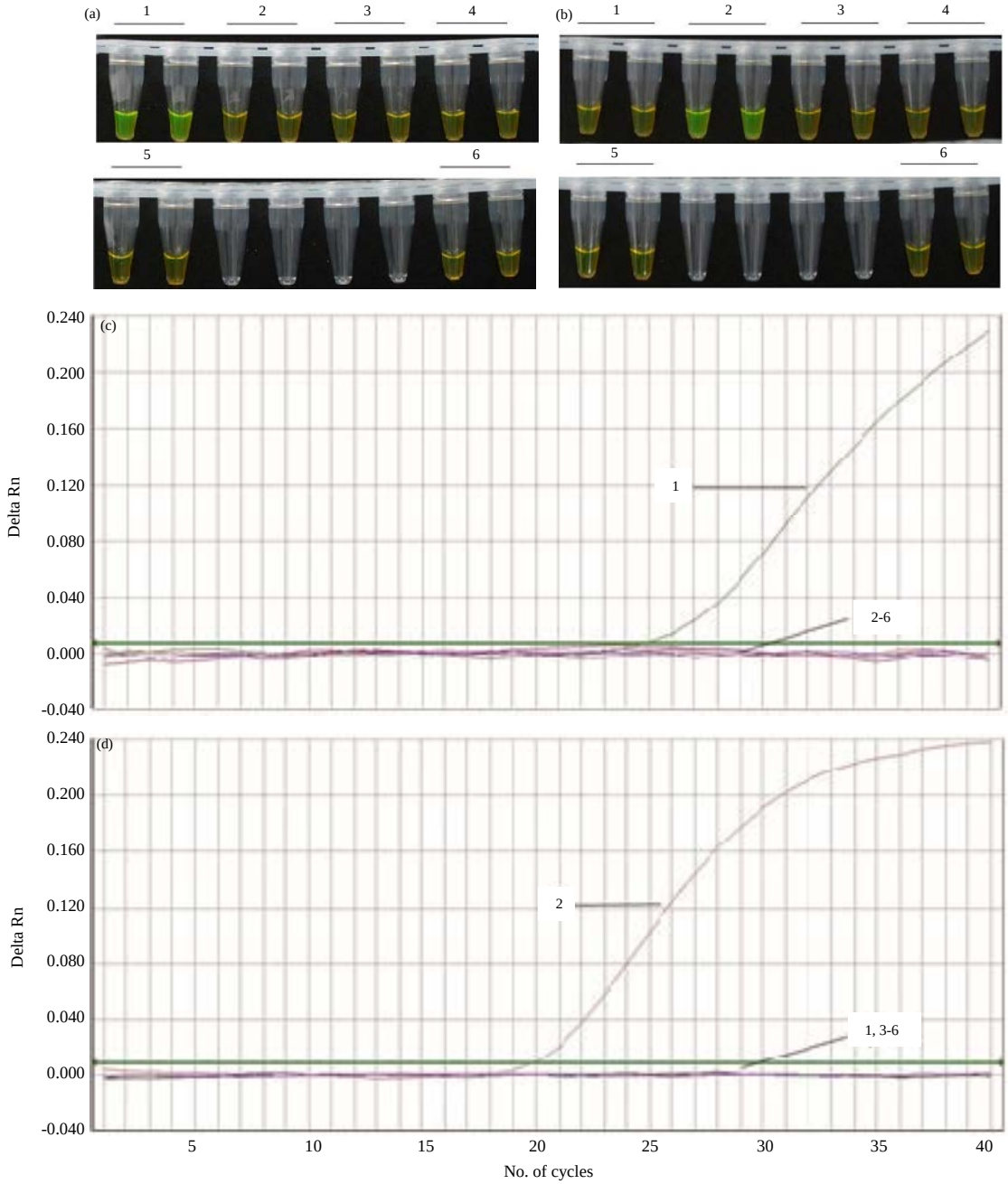

Fig. 2(a-d): Specificity analysis of the LAMP assay and real-time PCR, (a and b) LAMP detection for *Bos taurus* and *Ovis aries* and (c and d) Real-time PCR for *Bos taurus* and *Ovis aries*, 1: Bovine, 2: Ovine, 3: Horse, 4: Pig, 5: Chicken and 6: No template control

perfectly in order to amplify the target sequence. The mitochondrial DNA mainly consisted of coding sequence, which had the strict conservative but also had high variability among different species. The CoI and Cyt b gene were chosen as the specific fragment. Therefore, it confirmed that the primers had high species-specificity with the panel of species investigated.

Detection limit of visual LAMP assay: In the practical application of detection, the most of samples contained low quantity DNA. The high sensitivity was important and necessary in animal-derived ingredients detection. In the sensitivity experiment, the genomic DNA was supported in sensitivity and linearity of the LAMP technique with the initial concentration as 2.0 ng μL^{-1}. The template

concentration was diluted with TE buffer by 10 times and the final concentrations were ranged from 2.0×10^{0} to 2.0×10^{-7} ng μL^{-1}. Every test was repeated three times. To ensure the reliability of these experimental data, the qPCR assay was used as a reference.

The decreasing concentration indicated that the detection limit for bovine and ovine by visual LAMP assay were 2.0×10^{-3} ng μL^{-1}, respectively (Fig. 3a, b). The real-time PCR could detect 2.0×10^{-5} ng μL^{-1}, respectively (Fig. 3c, d). The detection limit of visual LAMP assay was on the same order of magnitude as previous studies[13-15]. The result showed the real-time PCR had higher sensitivity than LAMP about 2 orders of magnitude.

LAMP analysis of bovine and ovine in meat mixtures: For testing the detect ability of bovine and ovine in meat products, the LAMP method was used to analyze heat-treated beef mixtures containing other meat at 0.0001, 0.001, 0.01, 0.1, 1 and 10%. The mutton mixtures were carried out with the similar steps. Every ratio test repeated three times.

The positive results observed from the color change, showed the tested levels could reach 0.01 and 0.001% on bovine and ovine, respectively (Fig. 4a, b). The amplified curve of qPCR reflected the tested levels could reach 0.0001%, respectively (Fig. 4c, d). These data were consistent with the results for the detect ability of the genomic DNA before.

In this study, the visual LAMP assay allowed the detection of as few as 5.0×10^{-3} ng of bovine and ovine genomic DNA, respectively. Sensitivity levels resulted in bovine 0.01% of meat mixtures and in ovine 0.001%, respectively. Although, it was lower than the detect ability of the Taqman real-time PCR, the visual LAMP assay was 10 times higher than the standard PCR[3,18]. The method had the same sensitivity as the SYBR Green real-time PCR[19,20]. Furthermore, the outstanding advantages of the visual LAMP technology were fast and convenient. Thus, it could be used as a preliminary screening tool for detecting meat samples, greatly reducing the workload and cost for detection.

Tests of practical samples: The applicability of the visual LAMP assay for 489 blind samples had been demonstrated. All the samples were also analyzed by real-time PCR assay as a standard reference. There were 465 positive animal-derived samples been detected and the positive rate was 95.09% (Table 2). Compared to domestic sales of raw meat and commercial products, the detection rate is completely

Table 2: Results of the detection for the bovine and the ovine-derived component in blind samples

Animal-derived samples				Red meat and processed meat products							
Imported		Domestic		Commercial products		Raw meat					
Detected samples	Positive samples	Detected samples	Positive samples	Detected samples	Positive samples	Detected samples	Positive samples	Total detected samples	Total positive samples	Positive rate (%)	
18	17	471	448	26	3	445	445	489	465	95.09	

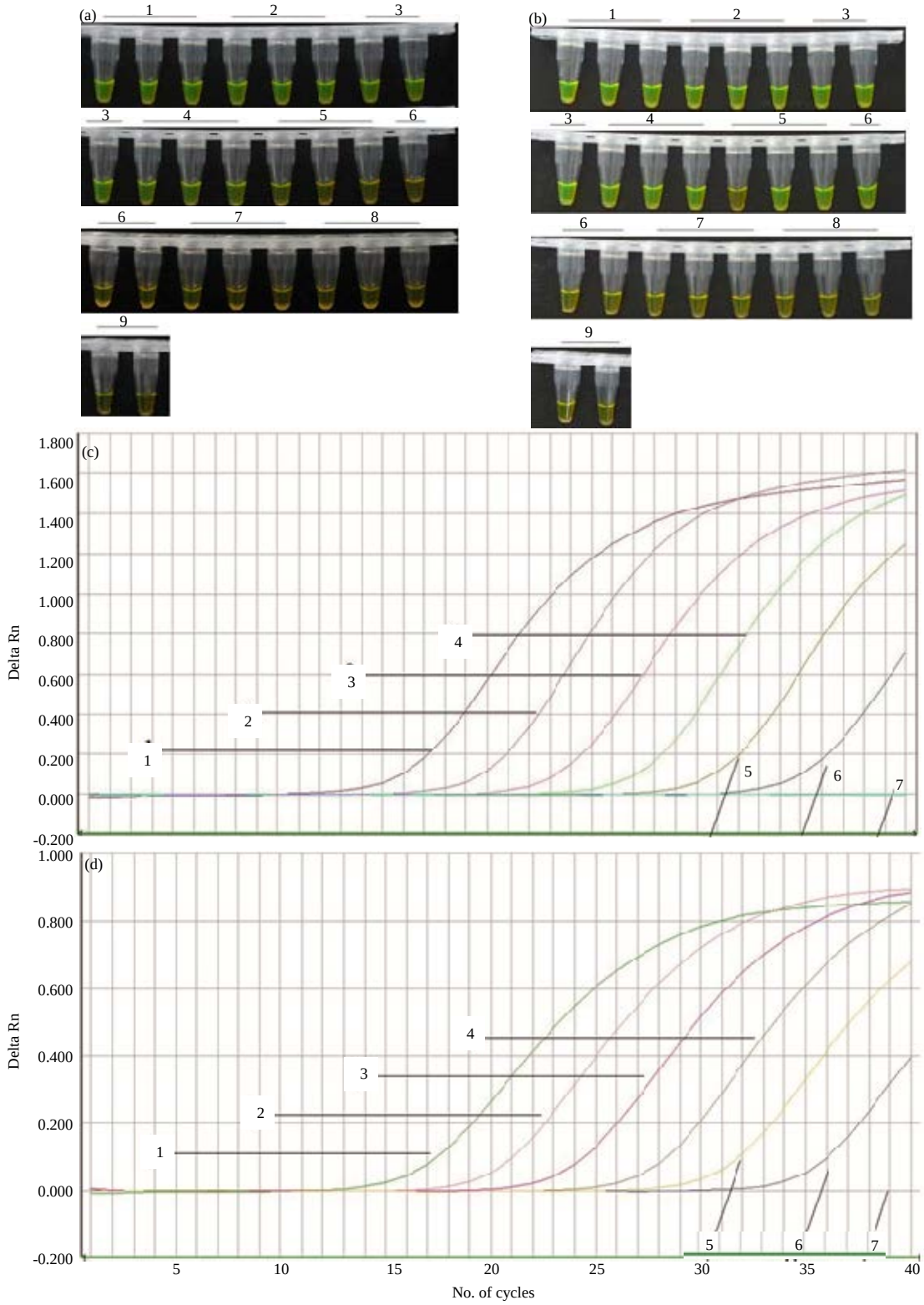

Fig. 3(a-d): Sensitivity analysis of the LAMP assay and real-time PCR, (a and b) LAMP detection for *Bos taurus* and *Ovis aries*; 1: 2.0 ng μL^{-1}, 2: 2.0×10^{-1} ng μL^{-1}, 3: 2.0×10^{-2} ng μL^{-1}, 4: 2.0×10^{-3} ng μL^{-1}, 5: 2.0×10^{-4} ng μL^{-1}, 6: 2.0×10^{-5} ng μL^{-1}, 7: 2.0×10^{-6} ng μL^{-1}, 8: 2.0×10^{-7} ng μL^{-1}, 9: Negative template control and (c and d) Real-time PCR for *Bos taurus* and *Ovis aries*; 1: 2.0 ng μL^{-1}, 2: 2.0×10^{-1} ng μL^{-1}, 3: 2.0×10^{-2} ng μL^{-1}, 4: 2.0×10^{-3} ng μL^{-1}, 5: 2.0×10^{-4} ng μL^{-1}, 6: 2.0×10^{-5} ng μL^{-1}, 7: No template control

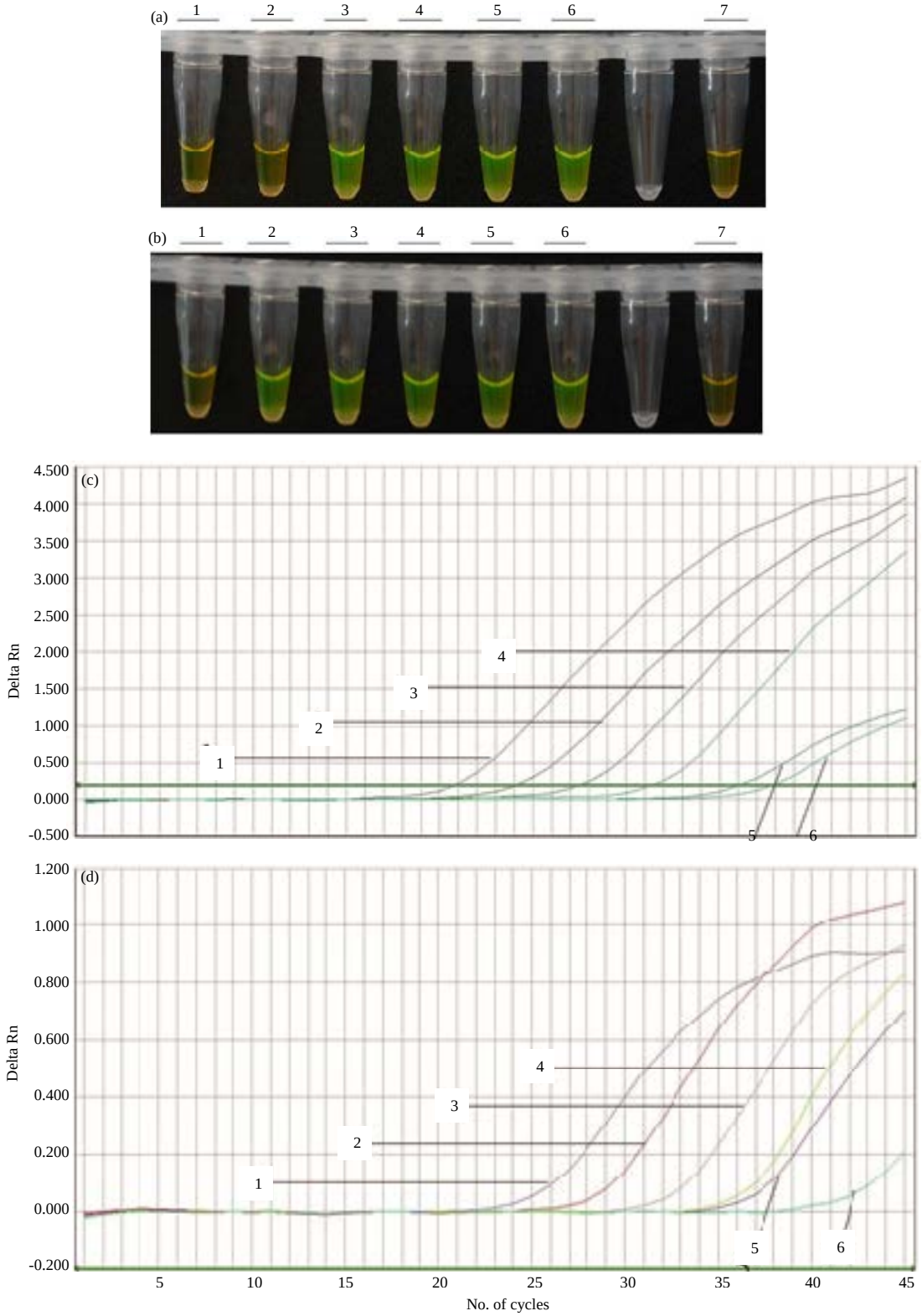

Fig. 4(a-d): The detection limit of the LAMP assay and real-time PCR in meat mixtures, (a and b) LAMP detection for *Bos taurus* and *Ovis aries*; 1: 0.0001, 2: 0.001, 3: 0.01, 4: 0.1, 5: 1.0, 6: 10, 7: No template control and (c and d) Real-time PCR for *Bos taurus* and *Ovis aries*; 1: 0.1, 2: 0.01, 3: 0.001, 4: 0.0001, 5: 0.00001, 6: No template control

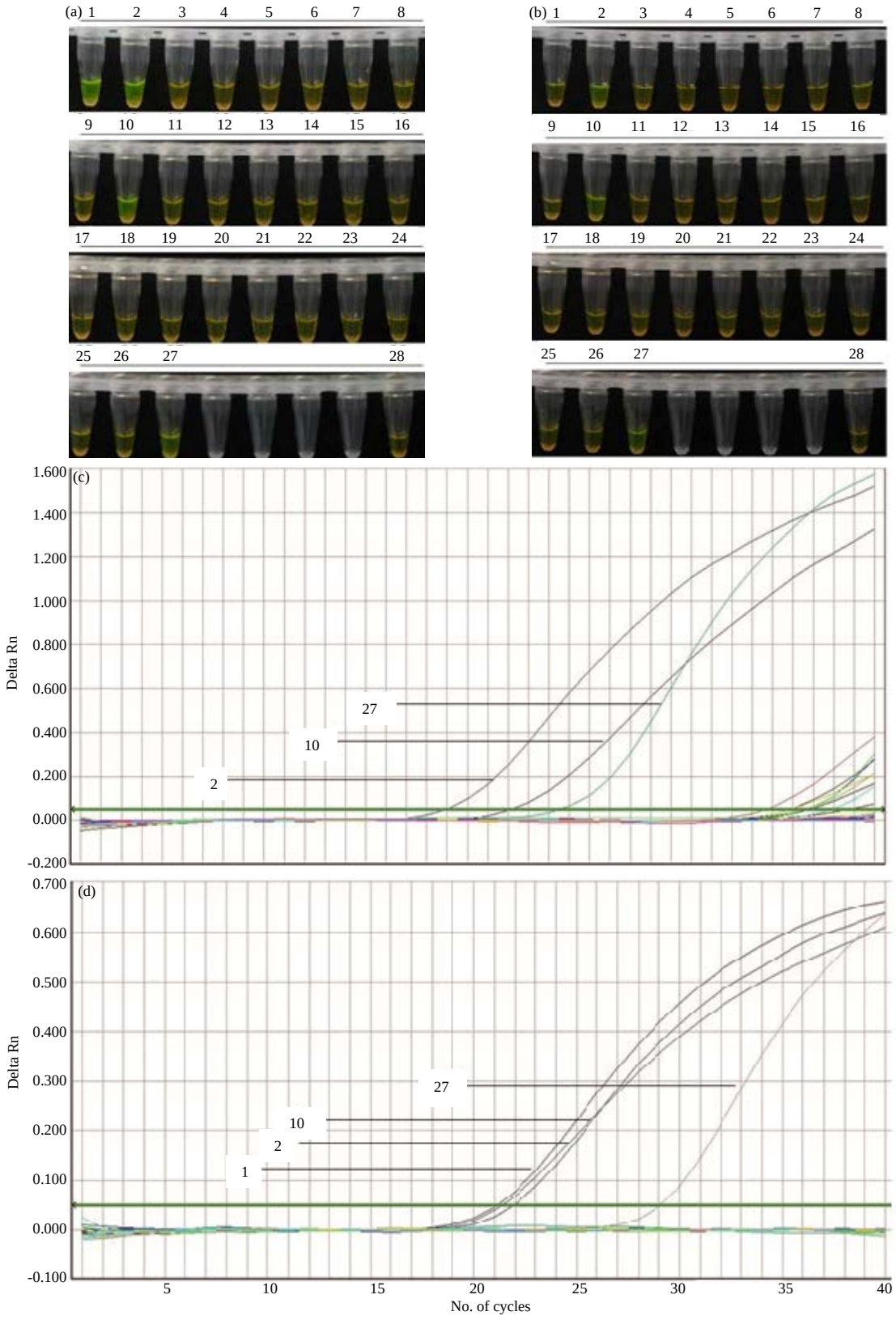

Fig. 5(a-d): Results of the LAMP assay and real-time PCR for detecting 26 unknown samples in commercial meat products, (a and b) LAMP detection for *Bos taurus* and *Ovis aries* and (c and d) qPCR detection for *Bos taurus* and *Ovis aries*; 1-26: Blind commercial samples, 27: Positive control, 28: No template control

Table 3: Concordance between LAMP and qPCR tests in 26 commercial products for detection of the bovine and the ovine-derived component

	Bos taurus		*Ovis aries*	
Products No.	LAMP	qPCR	LAMP	qPCR
86[a]	-	-	+	+
138[a]	+	+	+	+
151[a]	-	-	-	-
154[a]	-	-	-	-
156[a]	-	-	-	-
161[a]	-	-	-	-
162[a]	-	-	-	-
171[a]	-	-	-	-
582[a]	-	-	-	-
727[a]	+	+	+	+
889[a]	-	-	-	-
J-1301[a]	-	-	-	-
J-1302[a]	-	-	-	-
J-1303[a]	-	-	-	-
ZD-1[a]	-	-	-	-
ZD-2[a]	-	-	-	-
ZD-3[a]	-	-	-	-
ZD-4[a]	-	-	-	-
ZD-A[a]	-	-	-	-
ZD-B[a]	-	-	-	-
ZD-C[a]	-	-	-	-
ZD-D[a]	-	-	-	-
ZD-E[a]	-	-	-	-
SSF[a]	-	-	-	-
DB-1103[a]	-	-	-	-
ZN-1204[a]	-	-	-	-
Positive control 1	+	+	-	-
Positive control 2	-	-	+	+
NTC	-	-	-	-

[a]Number of blind samples, NTC: No template control, +: Positive result and -: Negative result

different. All fresh meat samples have been detected to the related origin. The concordance between the LAMP assay and qPCR tests in 26 commercial products for detection of the bovine- and the ovine-derived component were shown (Table 3). According to the results, the bovine gene was detected in No. 138 and 727 samples and the ovine gene was tested in No. 086, 138 and 727 samples (Fig. 5). The results of the two methods are consistent with the rate of 100%. This saved time, simplified standardization and also increased the specificity, sensitivity, accuracy and repeatability. If it is combined with simple and fast DNA extraction methods, which would be further useful for meat detection in the laboratory and also on site.

CONCLUSION

The LAMP assay was capable of amplifying a DNA target under isothermal conditions and the amplification products can be visually detected. It could be performed in less than 1 h without special equipment. Furthermore, the naked eye visualization using calcein eliminated the need for a UV transilluminator. In conclusion, this method offered a great advantage for the rapid onsite testing of food products. It was considered as a powerful technology for the control of foodstuff, which was accurate, simple and economical, with good sensitivity and specificity.

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