Sensitive detection of chicken in commercial processed food products based on one-step colourimetric loop-mediated isothermal amplification

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Abstract

The incidence of meat adulteration has been increasingly reported and these circumstances have raised great concern for food quality and safety in food industries worldwide. Due to its cheaper value compared with other types of consumable meat, chicken tends to become a major source of adulteration in meat products. The main objective of our present research was to develop a new detection platform based on loop-mediated isothermal amplification (LAMP) for authenticating chicken in both raw meat materials and processed food products. The optimal condition for colourimetric LAMP was investigated in order to achieve the highest yield of amplified LAMP products within the shortest period of time. Neutral red, a pH-sensitive indicator, was introduced into LAMP reactions to allow the positive/negative outcome to be distinguished. The LAMP reactions containing amplified LAMP amplicons resulted in the change of neutral red colour to pink/magenta while the reactions without amplified DNA products remained in their original yellow colour. Colourimeric LAMP can be rapidly completed in one step without the need to add additional reagents. The assay has been proven for its high specificity to chicken DNA without cross-reactivity with DNA from the other meat species. In addition, the assay was highly sensitive with the limit of detection (LOD) for chicken DNA as low as 1 pg and the LOD for chicken in binary meat mixtures of 0.01% (w/w). Tested with 28 commercial processed food samples, the assay confirmed the presence of chicken content in 14 chicken-containing products, and identified chicken content in 7 non-chicken products. With its simplicity of use, cost effectiveness, and rapidity, we anticipated that the assay developed could be a valuable analytical tool to support on-site services and low resource laboratory settings as parts of food authentication.

Keywords

Loop-mediated isothermal amplification; meat adulteration; chicken adulteration; colourimetric LAMP; food safety

Research highlights

- A new analytical platform for authentication of chicken based on colourimetric loop-mediated isothermal amplification technique has been successfully developed.
- The method was fast-processing with the overall time of 1 h and the detection outcome could be observed immediately as soon as the LAMP reactions terminated.
- The assay was highly specific to chicken DNA without cross-reactivity with the other meat species, including closely-related species.
- This approach offered high sensitivity of detection with the LOD for chicken DNA as low as 1 pg and the LOD for chicken content in binary meat admixtures was 0.01% (w/w).

- The LAMP assay developed has presented its applicability of identifying chicken content in commercial processed food products.

1. Introduction

In 2013, there were the scandals involving reports of beef products adulterated by horse meat, which was not declared on the product labels in the European region (O'Mahony, 2013). This event has adversely affected consumers' trust on meat products and raised great concern for food fraud, safety and quality in the food industry. In the past decade, incidences of food adulteration have increasingly been reported (Di Pinto et al., 2015; Kane & Hellberg, 2016; Premanandh, 2013). One of the fraudulent practices is partial or full substitution of higher commercial valued meat types with lower commercial valued ones without declaration of their presence to reduce the cost of production and increase profits (Barakat et al., 2014; Cawthorn et al., 2013). In addition, another source of food adulteration could be from accidental cross-contamination with undeclared meat species or ingredients as a result of improper hygiene in handling and cleaning equipment (Keyvan et al., 2017; Omran et al., 2019). In meat products, chicken, one of the most consumed meats, tends to be a main adulterant since its price is cheaper as compared to other meats. The incidence of chicken adulteration has been investigated and found in many commercial processed meat products (Keyvan et al., 2017; Kim & Kim, 2019; Kitpipit et al., 2014), often in blended meats. For the purpose of accurate identification and verification of chicken content in meat products, development of efficient analytical approaches is necessary in order to gain consumers' trust and promote fair trades in food markets locally and internationally.

A number of analytical techniques based on various principles including electrophoresis, chromatography, immunology, and mass spectrometry have been successfully developed for meat species specification (Alikord et al., 2018; Ballin et al., 2009; Montowska & Pospiech, 2010). Despite their specificity to meat species, these techniques still present limitations with low sensitivity and unsuitability for complex and processed food samples (Alikord et al., 2018). DNA-based methods are thus considered to be superior for meat species detection since DNA is highly thermally stable (Ballin, 2010; Nešić et al., 2017) whereas protein can easily be denatured at high temperature and pressure (Alikord et al., 2018). Polymerase chain reaction (PCR) – based techniques such as species-specific PCR, randomly amplified polymorphic DNA-PCR (RAPD-PCR), restriction fragment length polymorphism-PCR (RFLP-PCR), real-time PCR and digital PCR have been applied for identification of meat species with great sensitivity and specificity to their targets (Alikord et al., 2018; Kumar et al., 2015). However, the requirements of special experimental devices, expensive reagents, and skilled operators limit the applicability of the techniques to sophisticated laboratory settings (Ali et al., 2012; Ckumdee et al., 2016; Seetang-Nun et al., 2013). These limitations pave the way for new bioanalytical approaches for meat species authentication.

Loop-mediated isothermal amplification (LAMP), first introduced in 2000, is a novel DNA amplification method (Notomi et al., 2000). LAMP relies on the strand displacement activity of a specific enzyme e.g. *Bst* or *Bsm* DNA polymerase (Wang et al., 2019) and the amplification of DNA can be achieved at a constant temperature with 2-3 pairs of primers designed from 6-8 distinct regions of the target DNA sequences (Notomi et al., 2000; Tomita et al., 2008). In general, ladder-like patterns of amplified DNA are created as final products from LAMP, which can be analysed using gel electrophoresis (Notomi et al., 2000). During LAMP, magnesium pyrophosphate is formed as a by-product and this results in white insoluble salts that can be visually observed after termination of the reactions (Mori et al., 2001). However, the white precipitation is sometimes difficult to visually observe and may be ambiguous for interpretation of results (Tomita et al., 2008). Combining LAMP with other detection systems based on the difference of positive and negative reaction colours visible to the naked eye could be a good solution. Some reagents and nanoparticles have been successfully used in combination with LAMP, e.g. gold nanoparticles, whose colour change from red to purple/colourless upon particle aggregation (Ckumdee et al., 2016; Seetang-Nun et al., 2013; Thangsunan et al., 2021), and hydroxy naphthol blue (HNB), where the change of colour from purple to sky blue is observed as a consequence of the alteration of magnesium ion concentration during LAMP reactions (Duan et al., 2014; Goto et al., 2009; Nie et al., 2013; Yang et al., 2014). pH-sensitive indicators such as phenol red, cresol red, neutral red and m-cresol purple can also be employed with LAMP to monitor the successful amplification of DNA products as hydrogen ions are produced during LAMP, significantly changing the pH from initial alkaline to final acidic (Tanner et al., 2015; Wang et al., 2019; Xiong et al., 2020). This use of colour change to reveal positive and negative outcomes can be easily observed, and in the cases of using HNB and pH-sensitive dyes, the possibility of contamination from opening reaction tubes during experiments can also be eliminated.

Our work presented here was aimed at developing a new colourimetric LAMP assay for specific detection of chicken when present in raw meat materials and processed food products. A pH-sensitive indicator was used in LAMP reactions to identify the presence or absence of amplified LAMP amplicons. The developed assay was cost-effective and easy-to-operate since it only requires a simple temperature-controllable lab device such as a water bath, a heating block or a hot air oven. We anticipate that this assay could be a promising alternative for monitoring chicken content in real chicken-containing products and chicken as an adulterant in other non-chicken products in food.

2. Materials and methods

2.1 Sample preparation and DNA extraction

Fresh chicken (*Gallus gallus*), duck (*Anas platyrhynchos*), quail (*Coturnix coturnix*), pork (*Sus scrofa*), beef (*Bos Taurus*), sheep (*Ovis aries*), salmon (*Salmo salar*), deer (*Rusa unicolor*) and crocodile meat (*Crocodylus siamensis*) were purchased from supermarkets in Pathum Thani, Thailand. Fresh turkey (*Meleagris gallopavo*) was obtained from a local farm located in Maha Sarakham, Thailand. All the fresh meat samples were separately blended and lyophilized using a freeze-dryer (CHRIST, Gamma 1-16 LSC, Germany). The dried meat powder was then sieved through a 300-μm standard test sieve (Retsch, Fisher Scientific, USA) and stored at -80 ^{o}C until further use.

Binary meat admixtures of chicken and non-chicken meats (turkey, crocodile meat, pork or beef) were prepared. Lyophilized powder of chicken was mixed with one of the non-chicken meats selected previously at ratios of 100, 10, 1, 0.1, 0.01 and 0% (w/w), respectively. The prepared mixtures were stored at -80 ^{o}C until further use.

Twenty-eight commercially available processed meat products including 14 samples of chicken-containing products and 14 samples of non-chicken products as declared on the product labels were purchased from supermarkets and online retail stores in Pathum Thani, Bangkok and Lamphun, Thailand. All the processed products were stored at -20 ^{o}C until further use without freeze-drying.

To obtain genomic DNA for further studies, 200-500 mg of lyophilized meat powder or ground processed food products was extracted and purified using DNeasy Mericon Food Kit (Qiagen, Germany) according to the manufacturer's instructions. The purified DNA samples were subsequently measured for their concentration and purity using a NanoDrop One UV-Vis spectrophotometer (Thermo Scientific, USA).

2.2 Positive control plasmid construction

The nucleotide sequence of the chicken cytochrome b gene (GenBank Accession No. AF028795.1) was retrieved from the National Center for Biotechnology Information (NCBI) database. The primers used for polymerase chain reaction are presented in Table 1. In the total volume of 25 µl, each PCR reaction contained 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 µM dNTP mix, 0.3 µM each of forward and reverse primers (Table 1), 10 ng chicken genomic DNA, and 2.5 U *Taq* DNA polymerase (Invitrogen, USA). The PCR cycle began with an initial denaturation at 94 ^{o}C for 3 min, followed by 35 cycles of denaturation at 94 ^{o}C for 45 s, annealing at 58 ^{o}C for 3 s and extension at 72 ^{o}C for 45 s, and ended with a final extension at 72 ^{o}C for 10 min. The analysis of PCR products was then conducted using 1.5% (w/v) agarose gel electrophoresis in 1x Tris-borate-EDTA buffer system. The PCR products were purified using a PCR clean-up kit (Macherey-Nagel, Germany) prior to plasmid construction.

For positive control plasmid construction, the 591 bp PCR fragment from the partial chicken cytochrome b gene was ligated into pCR® 2.1 vector (Invitrogen, USA) as recommended by the manufacturer. The constructed plasmid was then transformed to OneShot™ Chemically Competent

Escherichia coli (Invitrogen, USA) using a heat-shock protocol. The transformed bacterial cells were grown on LB agar with 100 µg/ml ampicillin. Some of the grown colonies were selected according to the blue-white colony selection protocol and cultured in LB broth containing 100 µg/ml ampicillin. The plasmids were then extracted using a QIAprep® Spin Miniprep kit (Qiagen, Germany) according to the manufacturer's instructions. The purified plasmids were determined for the concentration and purity using a NanoDrop One UV-Vis spectrophotometer (Thermo Scientific, USA) prior to confirming the correction of the inserted sequence by sequencing.

Table 1 Nucleotide sequences of the primers used in the study

Primer name	Sequence (5' to 3')	Number of bp
Primers for positive control plasmid construction (this work)		
Cytb-Gallus-F	ATGCACTACACAGCAGACACA	21
Cytb-Gallus-R	GAGGTTGGGGGAGAATAGGGCT	22
Primers for LAMP assay (this work)		
Cytb-Chic-FIP	TGATAACGGTGGCCCCTCAGAGCCTTTGTGGGCTATGTTCT	41
Cytb-Chic-BIP	TAGTAGAGTGAGCCTGAGGGGGAAGCGAAGAATCGGGTAAGG	42
Cytb-Chic-F3	ATCCTCCTCCTCACACTCAT	20
Cytb-Chic-B3	GGTGAGGTGGATGATAGTAATA	22
Primers for conventional PCR targeting chicken cytochrome b gene (Kim et al., 2018)		
Chicken-CYTB-F	AGCAATTCCCTACATTGGACACA	23
Chicken-CYTB-R	GATGATAGTAATACCTGCGATTGCA	25

2.3 LAMP primer design

The partial sequence of cytochrome b gene from chicken (GenBank Accession No. AF028795.1) inserted into the positive control plasmid was employed for LAMP primer design. Primer Explorer version 5 software used for primer design is online available at https://primerexplorer.jp/e/v5_manual/index.html. The details of primer locations and sequences are shown in Fig. S1 and Table 1. All of the primers for LAMP assays were synthesized and PAGE-purified by Macrogen Inc. (Korea).

2.4 Colorimetric LAMP assay

The colourimetric LAMP protocol for this work was modified from the previously published studies (Tanner et al., 2015; J. Wang et al., 2019). In a total volume of 12.5 µl, each LAMP reaction comprised 1x LAMP buffer containing 2.5 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 8 mM $MgSO_4$, 0.1% Triton® X-100 pH 8.8, 1.4 mM dNTP mix, 1.6 µM each of inner primers (Cytb-Chic-FIP and Cytb-Chic-BIP, Table 1), 0.2 µM each of outer primers (Cytb-Chic-F3 and Cytb-Chic-B3, Table 1), 0.4 M betaine (Sigma-Aldrich, USA), 180 µM neutral red (Sigma-Aldrich, USA), 4 U of *Bst* DNA polymerase (Large fragment; New England Biolabs Inc., Beverly, MA, USA), 10 ng of DNA template. Plasmid DNA containing chicken cytochrome b gene (pCR-Cytb-Chick plasmid – Fig. S2) was used as a positive control while sterile deionised H_2O was used as a no-template control (NTC). All the prepared LAMP reactions were incubated at 65 °C for 60 min, followed by 2 min incubation at 90 °C so as to inactivate the enzyme. The alternation of solution colour was visually observed and the LAMP products were further analysed using 1.5% (w/v) agarose gel electrophoresis in 1x TBE buffer. Each LAMP experiment was repeated in triplicate at least to assure the consistency of the outcome.

2.5 Primer-specific polymerase chain reaction

The primer-specific PCR was performed as a standard protocol to compare with the colourimetric LAMP. The sequences of primers used for PCR were obtained from the previous study (Kim et al., 2018) and presented in Table 1. Each 12.5 µl of PCR reaction contained 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 µM each of forward and reverse primers (Chicken-CYTB-F and Chicken-CYTB-R – Table 1), 1.25 U of *Taq* DNA polymerase (Invitrogen, USA) and 10 ng of DNA template. The pCR-Cytb-Chick plasmid (Fig. S2) was used as a positive control while sterile ddH_2O was used as a no-template control (NTC). The PCR reaction was performed using the condition as follows: initial denaturation at 94 °C for 3 min, 30 cycles of denaturation at 94 °C for 45 s, annealing at 63 °C for 30 s, extension at 72 °C for 30 s, and final extension at 72 °C for 10 min. The PCR products were then analysed using 2.0% (w/v) agarose gel electrophoresis in 1x TBE buffer. Each PCR experiment was repeated in at least triplicate.

3. Results and Discussion

3.1 Optimisation of colourimetric LAMP assays

Mitochondrial DNA (mtDNA) genes such as cytochrome b, 12S rRNA, 16S rRNA and D loop region are generally used as target genes for speciation of meats (Kumar et al., 2015; Montowska & Pospiech, 2010; Sul et al., 2019). The reasons for this use are that the degree of point mutation accumulated in mtDNA genes among different animal species are relatively higher compared to nuclear DNA, whereas the sequences of mtDNA evolves slowly in the same animal species since they are maternally inherited (Farag et al., 2015; Kumar et al., 2015; Wang et al., 2019). These features make them suitable molecular markers for authentication of meat species, especially for closely-related meat species. mtDNA genes exists in multiple copies in mitochondria (Koh et al., 2011; Kumar et al., 2015; Wang et al., 2019). The multiple copies of mtDNA genes contribute to achieving high sensitivity of detection. In this research, the cytochrome b gene was selected as a target for developing a colourimetric LAMP assay to identify chicken content.

Several parameters affecting colourimetric LAMP performance, which are melting temperature (T_m), concentration of Tris-HCl in LAMP buffer, concentration of neutral red and incubation time, were optimised in order to obtain the highest yield of amplified LAMP products within the shortest period of time. To optimise the melting temperature during performing LAMP assays, the LAMP reactions were incubated at different temperatures varying from 61 to 67 °C. A plasmid carrying the cytochrome b gene from chicken (pCR-Cytb-Chick plasmid – Fig. S2) was used as positive DNA template whereas sterile deionised H_2O was used as a no-template control (NTC). After performing the LAMP assay, identical ladder-like patterns of LAMP amplicons were observed at all temperatures tested (Fig. 1a). As *Bst* DNA polymerase works effectively at temperature between 60 and 65 °C (Notomi et al., 2000), a T_m of 65 °C was selected as the optimal temperature to perform further LAMP experiments.

To perform colourimetric LAMP, neutral red, a pH-sensitive dye, was introduced into the LAMP reactions to allow visual observation of positive/negative outcome after performing the assays. During the incubation of LAMP reactions, a large amount of H^+ was generated, leading to a decrease in pH from alkaline to acidic (Tanner et al., 2015; Xiong et al., 2020). In the presence of a pH-sensitive indicator, the change of pH in the LAMP reactions can be monitored by the transition of its colour (Tanner et al., 2015; Xiong et al., 2020). In our work, the optimal concentration of Tris-HCl in LAMP buffer was investigated by varying its working concentration from 20 down to 2.5 mM. We found that at Tris-HCl concentrations between 20 and 10 mM, a slight change of LAMP reaction colour from yellow to light orange was seen in the reaction containing pCR-Cytb-Chick plasmid while no change in colour was observed for the NTC reaction (Fig. 1b). However, when decreasing the concentrations of Tris-HCl to 5 and 2.5 mM, a significant change of LAMP reaction colour from yellow to pink/magenta was detected in the positive control reaction (Fig. 1b). This outcome agrees with previous studies which recommended use of weakly buffered or non-buffered LAMP solutions as this can provide the possibility of successful detection using pH-sensitive indicators (Niessen et al., 2018; Tanner et al., 2015; Xiong et al., 2020). In this work, using 2.5 mM Tris-HCl gave the most significant change in LAMP reaction colour for positive detection and it was chosen as the optimal concentration of Tris-HCl for performing colourimetric LAMP. We also examined the optimal concentration of neutral red used as an indicator for monitoring positive/negative detection. The concentrations of neutral red were varied from 120 to 210 µM. It was observed in Fig. 1c that the change of pH-indicator colour for LAMP in the reaction with the positive control plasmid was clearly seen in all the neutral red concentrations tested. In this work, 180 µM neutral red was selected as the optimal concentration used to conduct further LAMP assays.

The optimal period of incubation for LAMP assays was also studied. Neutral red at the optimal concentration (180 µM) was added into the LAMP reactions in order to monitor the successful amplification of LAMP DNA products. During the LAMP assay, the T_m was constantly set at 65 ^oC with the incubation time varying from 30 to 75 min. It is shown in Fig. 1d that the change of the reaction colour from yellow to pink could be observed in the reaction containing pCR-Cytb-Chick plasmid when being incubated for 30 min, indicating positive detection. When the reaction was performed for longer periods of time (45-75 min), the original colour of yellow was turned to bright pink or magenta for the reactions with pCR-Cytb-Chick plasmid whereas the reactions without the plasmid retained in their original yellow colour (Fig. 1d). Data obtained from agarose gel electrophoresis were in good agreement with the results from visual observation as the increasing yield of LAMP products could be seen upon increasing incubation time (Fig. 1e), which is related to the change of LAMP reaction colour (Fig. 1d). Even though the reaction colour change could be detected at 30 min of incubation, we chose 60 min as the optimal incubation period so as to ensure the consistency of the LAMP assay performance, as it was the point where the colour change became consistent.

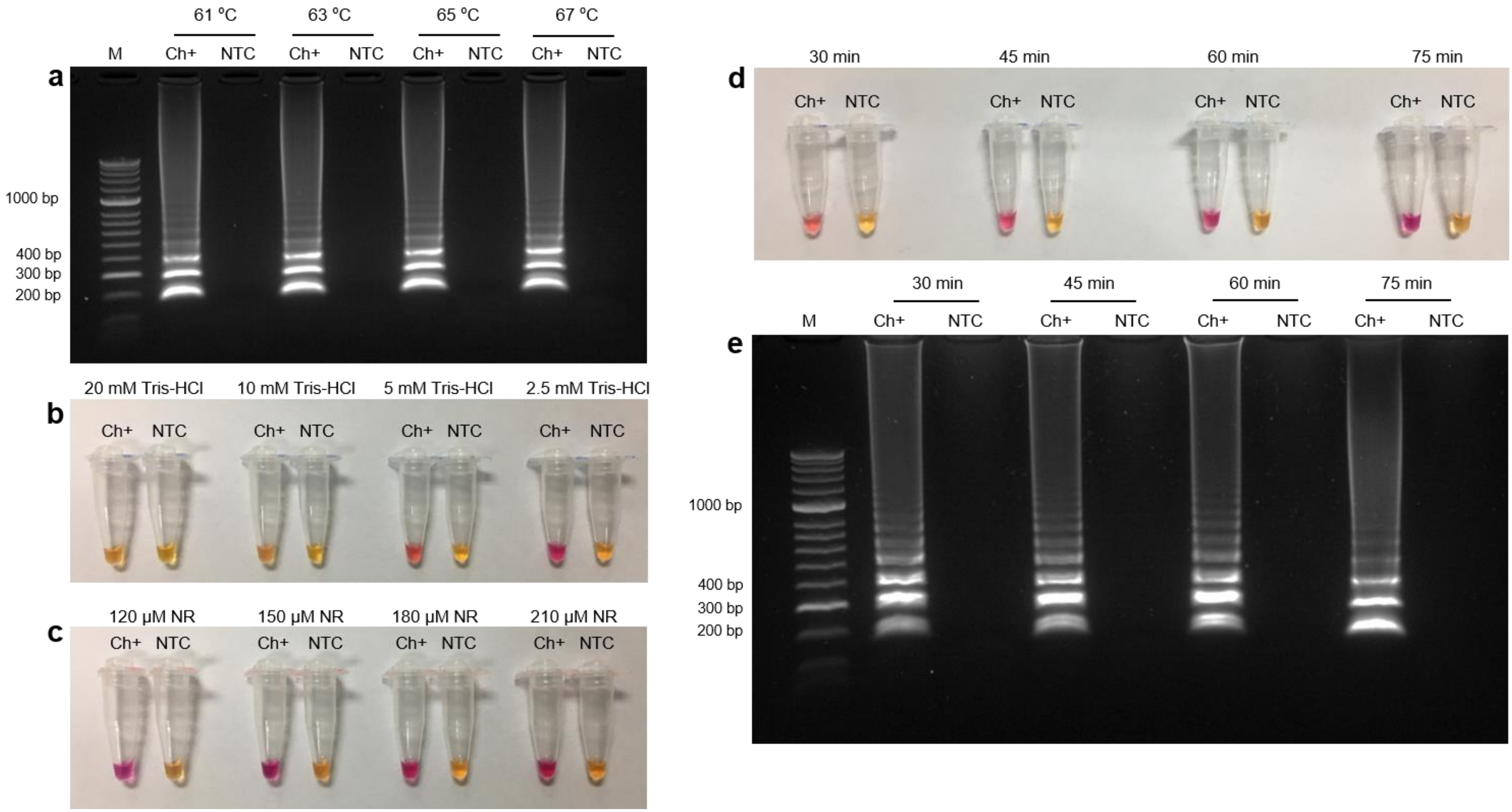

Fig. 1 Optimisation of factors affecting colourimetric LAMP performance for detection of chicken. (a), melting temperature (T_m) was varied in the range of 61 - 67 ºC. The LAMP products were analysed using 1.5% agarose gel electrophoresis; (b), the optimal concentration of Tris-HCl in LAMP buffer was tested by varying it from 20 mM down to 2.5 mM; (c), the optimal concentration of neutral red (NR) as an indicator positive/negative reactions was also examined by varying it from 120 to 210 µM; (d), the optimal LAMP period was studied by varying incubation time from 30 to 75 min; (e), LAMP amplicons from the reactions incubated for different periods of time were analysed using agarose gel electrophoresis. (M), HyperLadder 50 bp DNA marker (Bioline, UK); (Ch+), pCR-Cytb-Chick plasmid; (NTC), deionised H_2O as a no-template control.

3.2 Specificity of colourimetric LAMP assay

The specificity of the colourimetric LAMP assay is highly important since there is a possibility of cross reactivity, leading to false positives, especially when the assay is used with closely-related meat species. In our study, the LAMP assay for chicken was tested for its specificity against turkey, duck, quail, pork, beef, sheep, salmon, deer and crocodile. For visual observation, the reaction colour change from yellow to magenta was only detected in the reactions containing pCR-Cytb-Chick plasmid (positive control) and chicken gDNA while the reactions containing gDNA from other meats remained yellow (Fig. 2a). The data from agarose gel electrophoresis supported those obtained by visual observation as there were only the reactions with pCR-Cytb-Chick plasmid and chicken DNA showing the ladder-like pattern of amplified DNA amplicons in the agarose gel (Fig. 2b).

Conventional PCR, targeting cytochrome b gene of chicken, was used as a standard method to compare the specificity with the colourimetric LAMP assay developed here. The sequences of primers used to perform PCR were obtained from the previously published work (Kim et al., 2018). PCR was tested with the same gDNA from the meats used in the colourimetric LAMP assay. The result from agarose gel electrophoresis showed that 133-bp PCR products were detected in the reactions with pCR-Cytb-Chick plasmid (positive control) and chicken DNA samples (Fig. 2c) whereas no PCR products were amplified in the reactions containing DNA from the other meat species tested. This confirmed the specificity of the colourimetric LAMP assay in this work was comparable to the conventional PCR method. In addition, the specificity of the LAMP assay developed here was comparable to the previous LAMP detection methods capable of distinguishing chicken from other meat species including LAMP plus lateral flow dipstick targeting cytochrome b gene (Wang et al., 2020), LAMP plus annealing curve analysis targeting mitochondrial 16S rRNA gene (Sul et al., 2019), LAMP plus annealing curve analysis targeting ATP synthase F0 subunit 8 and subunit 6 genes (Cho et al., 2014) andLAMP plus electrochemical DNA sensor targeting mitochondrial 12S rRNA gene (Ahmed et al., 2010).

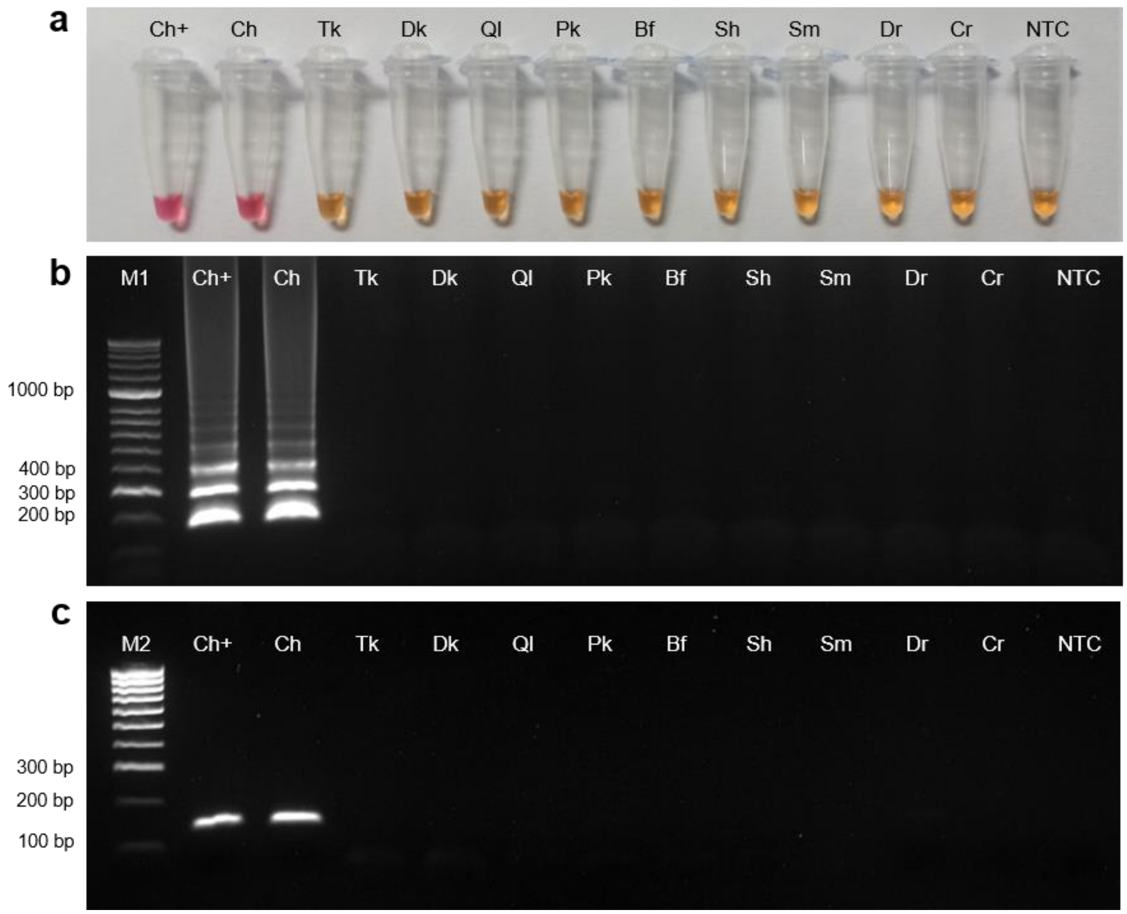

Fig. 2 Specificity of colourimetric LAMP and conventional PCR assays tested with genomic DNA extracted from different meat species. (a), the change of LAMP reaction colour after performing LAMP assay; (b), LAMP DNA products analysed by 1.5% (w/v) agarose gel electrophoresis and (c), DNA samples tested with the conventional PCR as a standard method for comparison, PCR products were analysed using 2.0% (w/v) agarose gel electrophoresis. (M1), HyperLadder 50 bp DNA marker (Bioline, UK) and (M2), HyperLadder 100 bp DNA marker (Bioline, UK). The assays were tested with a wide range of meat species including (Ch), chicken; (Tk), turkey; (Dk), duck; (Ql), quail; (Pk), pork; (Bf), beef; (Sh), sheep; (Sm), salmon; (Dr), deer and (Cr), crocodile. (Ch+), pCR-Cytb-Chick plasmid was used as a positive control and (NTC), deionised H_2O was used as a no-template control.

3.3 Sensitivity of colourimetric LAMP assay

In this work, the sensitivity of the colourimetric LAMP assay was also determined. The limit of detection for the LAMP assay is the lowest concentration of chicken DNA where the assay can still show positive detection. Chicken genomic DNA as a template DNA for the colourimetric LAMP assay was prepared for the concentrations ranging from 10 ng down to 1 fg by 10-fold dilution. The LAMP assay was performed at 65 ^{o}C for 60 min. The data from visual observations revealed that with chicken DNA from 10 ng down to 1 pg, the change of the reaction colour from yellow to magenta could be detected, indicating positive detection (Fig. 3a). However, at lower concentrations of chicken DNA (0.1 pg down to 1 fg), the reaction colour was unchanged (Fig. 3a). Agarose gel electrophoresis was used to analyse the amplified LAMP DNA products and it was found that the ladder-like pattern of LAMP products was seen in the reactions with the chicken DNA concentrations from 10 ng down to 1 pg (Fig. 3b). These data supported the results from visual observation and this showed that the limit of detection of chicken DNA for the colourimetric LAMP assay developed herein was as low as 1 pg of chicken DNA.

As a comparison, PCR with the primers specific to chicken cytochrome b gene (Kim et al., 2018). was used to test with the same concentrations of chicken genomic DNA. The data obtained from agarose gel electrophoresis revealed that the 133-bp PCR products were detected in the reactions containing pCR-Cytb-Chick plasmid and chicken DNA with the concentrations of 10 ng down to 10 pg (Fig. 3c). No PCR products were observed in the reactions with chicken DNA concentrations of 1 pg down to 1 fg (Fig. 3c). These data showed that the developed colourimetric LAMP assay was 10 times more sensitive than the conventional PCR used in this study. The sensitivity of the LAMP assay developed in this work was equal to or higher than that of the previously reported PCR-based methods (LOD range of 1 pg – 6.25 ng of DNA – Table 2) but this sensitivity was lower than that of RT-PCR-based methods (LOD of 0.1 pg of DNA – Table 2). In addition, our colourimetric LAMP assay (LOD = 1 pg DNA) was more sensitive than some of the previously reported LAMP methods (Table 2), which reported LODs between 5 to 78.68 pg of DNA (Ahmed et al., 2010; Cho et al., 2014; F. Wang et al., 2020). However, the work based on LAMP with annealing curve analysis targeting 16S rRNA gene showed the LOD for chicken DNA detection of 10 fg (Sul et al., 2019), which is more sensitive than our LAMP assay.

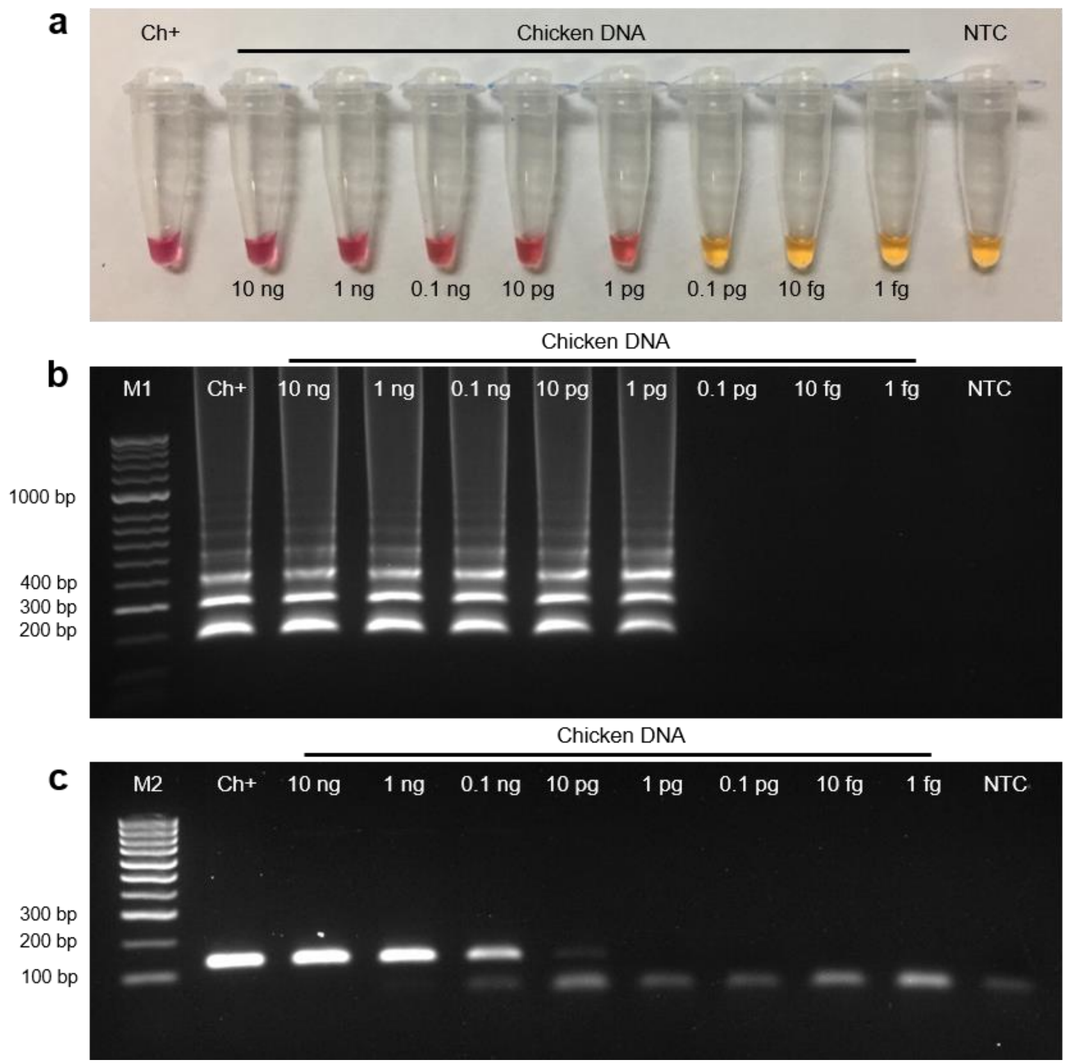

Fig. 3 Sensitivity of colourimetric LAMP and conventional PCR assays tested with different concentrations of chicken genomic DNA. Chicken DNA was prepared by 10-fold dilutions to obtain the working concentrations ranging from 10 ng down to 1 fg prior to performing the LAMP and PCR assays. (a), the change of the reaction colour after performing LAMP assay; (b), LAMP products from 1.5% (w/v) agarose gel electrophoresis and (c), PCR used as a standard method to detect different concentrations of chicken genomic DNA. The PCR products were analysed using 2.0% (w/v) agarose gel electrophoresis. (M1), HyperLadder 50 bp DNA marker (Bioline, UK); (M2), HyperLadder 100 bp DNA marker (Bioline, UK); (Ch+), pCR-Cytb-Chick plasmid as a positive control; and (NTC), deionised H_2O as a no-template control.

The sensitivity of the colourimetric LAMP assay was also determined in terms of percentage of chicken content in binary meat admixtures. Chicken genomic DNA as a template for LAMP was extracted from binary meat mixtures containing various ratio (100, 10, 1, 0.1, 0.01 and 0% (w/w)) of chicken in non-chicken meat background (turkey, crocodile, pork and beef). The reasons for choosing these four types of meat as the binary meat admixture backgrounds were that the texture and colour of fresh turkey and crocodile meat are similar to chicken while in commercial processed food products, chicken, pork and beef are three major meat species used for production and possibly adulterated with one another during manufacturing processes. After performing the LAMP assays, the reaction colour was changed from yellow to magenta in the samples of pCR-Cytb-Chick plasmid (positive control), 100%, 10%, 1%, 0.1% and 0.01% (w/w) of chicken in non-chicken meat admixtures (Fig. 4a-d (upper panels)). In the reactions with no chicken content, the solution remained yellow (Fig. 4a-d (upper panels)). The data from agarose gel electrophoresis revealed that in the LAMP reactions containing chicken DNA (100, 10, 1, 0.1 and 0.01% (w/w) of chicken in binary meat admixtures) and positive control plasmid, the ladder-like bands of amplified DNA products were generated whereas none of these bands were detected in the reactions with non-chicken DNAs and NTC (Fig. 4a-d (lower panels)). The outcomes obtained from visual observation (Fig. 4a-d (upper panels)) and electrophoresis (Fig. 4a-d (lower panels)) were in good agreement with each other. Thus, the detection limit obtained from the binary meat admixtures was as low as 0.01% (w/w) of chicken in meat admixtures.

As a standard method for comparison, PCR with the primers specific to chicken cytochrome b gene (Kim et al., 2018) was used to test its sensitivity with DNA extracted from the binary meat admixtures. In Fig. 4e – 4h, the results from agarose gel electrophoresis showed that the PCR products with the molecular size of 133 bp were seen in the binary meat admixtures containing 100, 10, 1, 0.1 and 0.01% (w/w) chicken no matter what type of non-chicken meat background was. As expected, 133-bp PCR products were also detected in the pCR-Cytb-Chick plasmid reaction, but not observed in the reactions of 0% chicken and NTC (Fig. 4e – 4h). The sensitivities of our colourimetric LAMP and standard PCR assays for chicken in the binary meat admixtures were in the same range of detection. The sensitivity of our colourimetric LAMP assay was also compared with the PCR and RT-PCR-based techniques and we found that our developed assay was more sensitive as the majority of those previously published PCR/RT-PCR based methods reported detection limits of 0.1 – 0.5% (w/w) chicken in other meat backgrounds (Table 2). However, two of the previous studies showed the detection limit of the assays as low as 0.001% (Fujimura et al., 2008; Kesmen et al., 2012), which was 10 times more sensitive than our LAMP method. Comparing the sensitivity of our colourimetric LAMP with other LAMP methods (Table 2), the colourimetric LAMP (LOD = 0.01% chicken) was more sensitive than the LAMP-LFD targeting chicken cytochrome b gene (LOD = 0.1% chicken) (Wang et al., 2020) and as sensitive as the LAMP with annealing curve analysis targeting ATP synthase F0 subunit 8 and 6 genes (LOD = 0.01% chicken) (Cho et al., 2014). As the presence of undeclared meat species under 0.1% in processed meat products is usually considered to be an unintentional adulteration with no economical purpose (Kesmen et al., 2012), the limit of detection for our colourimetric LAMP assay at 0.01% chicken would be adequate for applying with real processed food products.

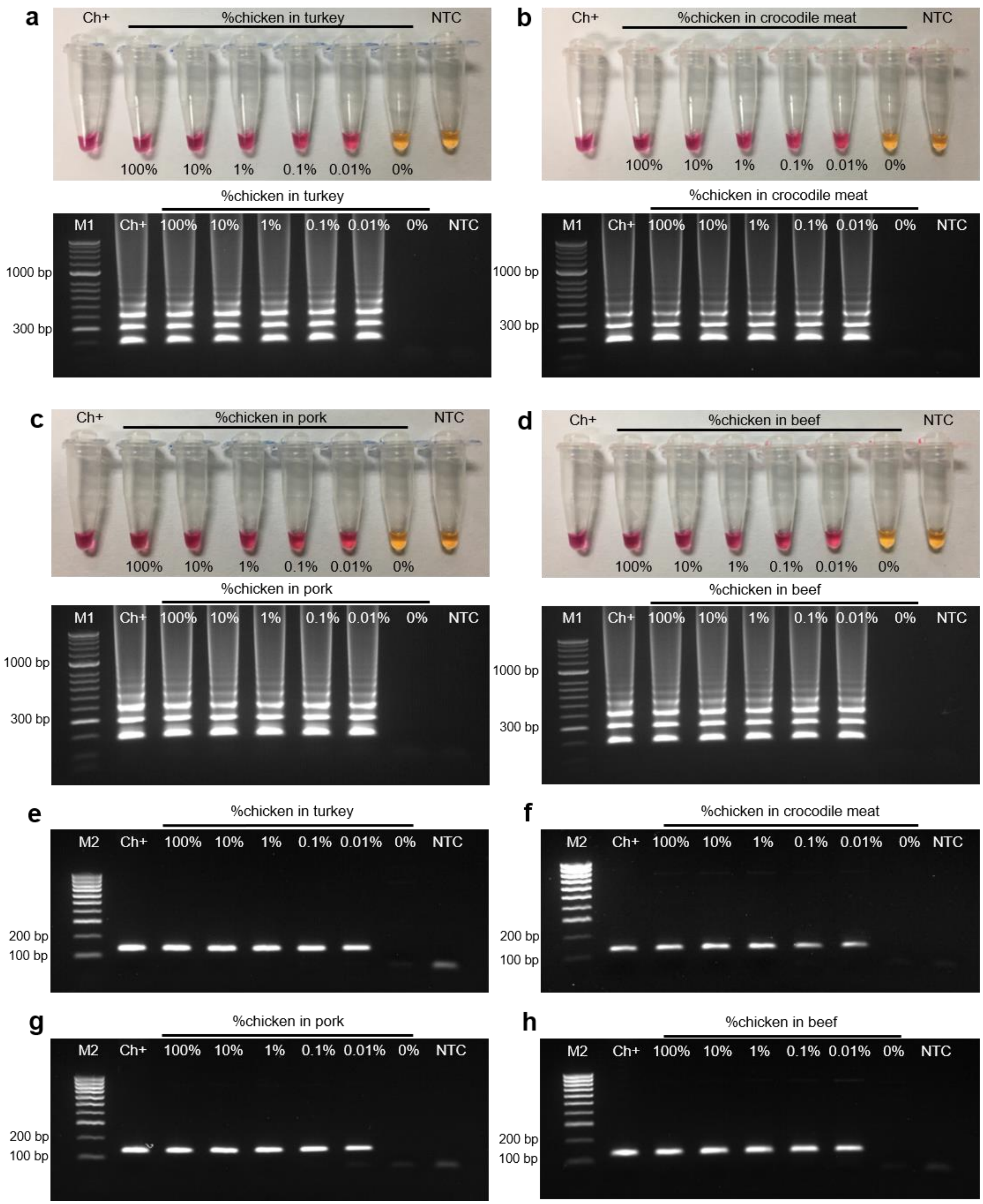

Fig. 4 Sensitivity of colourimetric LAMP assay tested with genomic DNA extracted from binary admixtures of chicken and non-chicken meats. The binary admixtures were prepared by mixing chicken

with another type of selected meat species with the ratio of 100, 10, 1, 0.1, 0.01 and 0% (w/w) of chicken in non-chicken meat background prior to DNA extraction. (a-d), the change of reaction colour after performing LAMP, and LAMP products analysed by 1.5% (w/v) agarose gel electrophoresis. (e-h), 2.0% (w/v) agarose gel electrophoresis showing the amplified products from conventional PCR as a standard method used to compare with colourimetric LAMP assay. Chicken was mixed with (a, e), turkey; (b, f), crocodile meat; (c, g), pork and (d, h), beef, respectively. (M1), HyperLadder 50 bp DNA marker (Bioline, UK); (M2), HyperLadder 100 bp DNA marker (Bioline, UK); (Ch+), pCR-Cytb-Chick plasmid as a positive control; and (NTC), deionised H_2O as a no template control.

Table 2 Sensitivity of DNA-based assays for detection of chicken

Methods	Target gene for chicken detection	Limit of detection of the assay	Reference
Specific PCR	12S rRNA gene	0.1% chicken in oat	(Martín et al., 2007)
Specific PCR	Nuclear 5-aminolevulinate synthase gene	0.1% chicken in meat matrix and 10 pg of chicken DNA	(Karabasanavar et al., 2013)
Multiplex PCR	Cytochrome b gene	1 pg of chicken DNA and 0.1% chicken in meat mixtures	(Kim et al., 2018)
Direct multiplex PCR	Cytochrome oxidase I subunit gene	12,500 mtDNA copies	(Kitpipit et al., 2014)
Nested-PCR	Cytochrome b and NADH dehydrogenase 5/6 genes	0.5 ng of chicken DNA	(Unajak et al., 2010)
Specific PCR	Mitochondrial D-loop gene	<1% chicken in admixed meat and meat products	(Mane et al., 2009)
Specific PCR	α-actin gene	0.1% chicken in foie gras	(Rodríguez et al., 2003)
Multiplex PCR	Cytochrome C oxidase subunit I gene	6.25 ng/µL of chicken DNA	(Izadpanah et al., 2018)
Specific PCR	16S rRNA gene	0.001% chicken in pork powder	(Fujimura et al., 2008)
Multiplex RT-PCR	Cytochrome b gene	0.1 pg of chicken DNA and 0.5% chicken in meat mixtures	(Kim & Kim, 2019)
RT-PCR	Cytochrome b gene	10 fg/µl of chicken mtDNA	(Tanabe et al., 2007)
RT-PCR	NADH dehydrogenase subunit 2 gene	0.1 pg of chicken DNA and 0.001% chicken in meat mixtures	(Kesmen et al., 2012)
LAMP with annealing curve analysis	16S rRNA gene	10 fg of chicken DNA and 0.1% chicken in meat mixtures	(Sul et al., 2019)s
LAMP-LFD	Cytochrome b gene	5 pg of chicken DNA and 0.1% chicken in beef	(Wang et al., 2020)
LAMP with electrochemical DNA sensor	12S rRNA gene	78.68 pg/µL of chicken DNA	(Ahmed et al., 2010)
LAMP with annealing curve analysis	ATP synthase F0 subunit 8 and 6 genes	10 pg/µL of chicken DNA and 0.01% chicken in meat mixtures	(Cho et al., 2014)

Colourimetric LAMP assay	Cytochrome b gene	1 pg of chicken DNA and 0.01% chicken in binary meat admixtures	This work

3.4 Testing colourimetric LAMP assay with commercial processed meat products

The colourimetric LAMP assay was tested for its practical uses with commercial processed meat products. Conventional PCR using the primers targeting chicken cytochrome b gene of chicken obtained from the previous study (Kim et al., 2018) was used as a standard method for comparison of the detection results. Twenty-eight commercial products (14 chicken-containing products and 14 non-chicken products) collected from supermarkets in the areas of Pathum Thani, Thailand and online stores in Thailand were examined for chicken content using both colourimetric LAMP and conventional PCR. Fig. 5 shows some of the randomly selected processed food samples. As expected, the LAMP reactions containing pCR-Cytb-Chick plasmid and the processed food samples declaring chicken content on their descriptions such as chicken ball-1 (sample no. 1), chicken Bolognese sausage (sample no.4) and chicken sausage-2 (sample no.7) tested positive for both visual observation (Fig. 5a) and agarose gel electrophoresis (Fig. 5b). These data agreed well with the result from the primer-specific PCR, which the 133-bp PCR products were observed in the same samples (Fig. 5c). Two non-chicken products, pork sausage-2 (sample no.16) and crocodile meatball (sample no.25), showed undetectable results for LAMP visual observation (Fig. 5a), LAMP agarose gel electrophoresis (Fig. 5b) and primer-specific PCR (Fig. 5c). Interestingly, some of the samples including pork sausage-3 (sample no.17), pork ball-1 (sample no.19) and beef ball-2 (sample no.22) were tested positive for both colourimetric LAMP (Fig. 5a and 5b) and PCR (Fig. 5c) in spite of no declaration of chicken content on the product labels, indicating chicken adulteration of the products. However, pork ball-2 (sample no.20) showed positive detection result for LAMP analysed using agarose gel electrophoresis (Figure 5b) while the LAMP reaction colour was slightly changed from yellow to orange (Fig. 5a), which was hardly detected by naked eye. The 133-bp PCR products were not observed for the pork ball-2 sample (Fig. 5c), which was opposite to the LAMP data. This could be explained by the previous experimental data showing that the sensitivity of the colourimetric LAMP was 10 times higher than that of the primer-specific PCR (Fig. 3); therefore, the LAMP assay was capable of detecting lower amount of chicken DNA adulterated in some processed food products.

The detection results for all the commercial processed meat products are presented in Table 3. Of all 28 samples tested, 14 products declaring the presence of chicken showed positive detection in both colourimetric LAMP and PCR assays. Seven samples with no declaration of chicken content showed undetectable results for both colourimetric LAMP and PCR whereas 5 non-chicken samples showed positive detection results for both assays even though chicken content was not declared on the product labels. Two non-chicken products, pork ball-2 (sample no. 20) and beef ball-1 (sample no.21), were tested positive for the colourimetric LAMP while were undetectable using the primer-specific PCR, confirming that the colourimetric LAMP assay is more sensitive than the PCR. Positive detection of chicken adulteration in the products without declaring chicken content could possibly be from an unintentional cross-contamination during manufacturing processes in cases where production lines for each meat species are not suitably separated and the machines used for production are not properly cleaned (Kim & Kim, 2019; Omran et al., 2019). In supermarkets, food products containing mixed pork and chicken, or mixed beef and chicken are often seen. Therefore, there is a high possibility of chicken adulteration in the products declaring the presence of only pork or beef if the equipment used for production is shared among different meat species. There have been the previous studies using PCR/RT-PCR methods to identify the contamination of chicken in pork and beef containing products (Kim et al., 2018; Kim & Kim, 2019; Kitpipit et al., 2014). Taken these data together, our colourimetric LAMP assay is capable of detecting chicken content in commercial processed meat products with high sensitivity and accuracy.

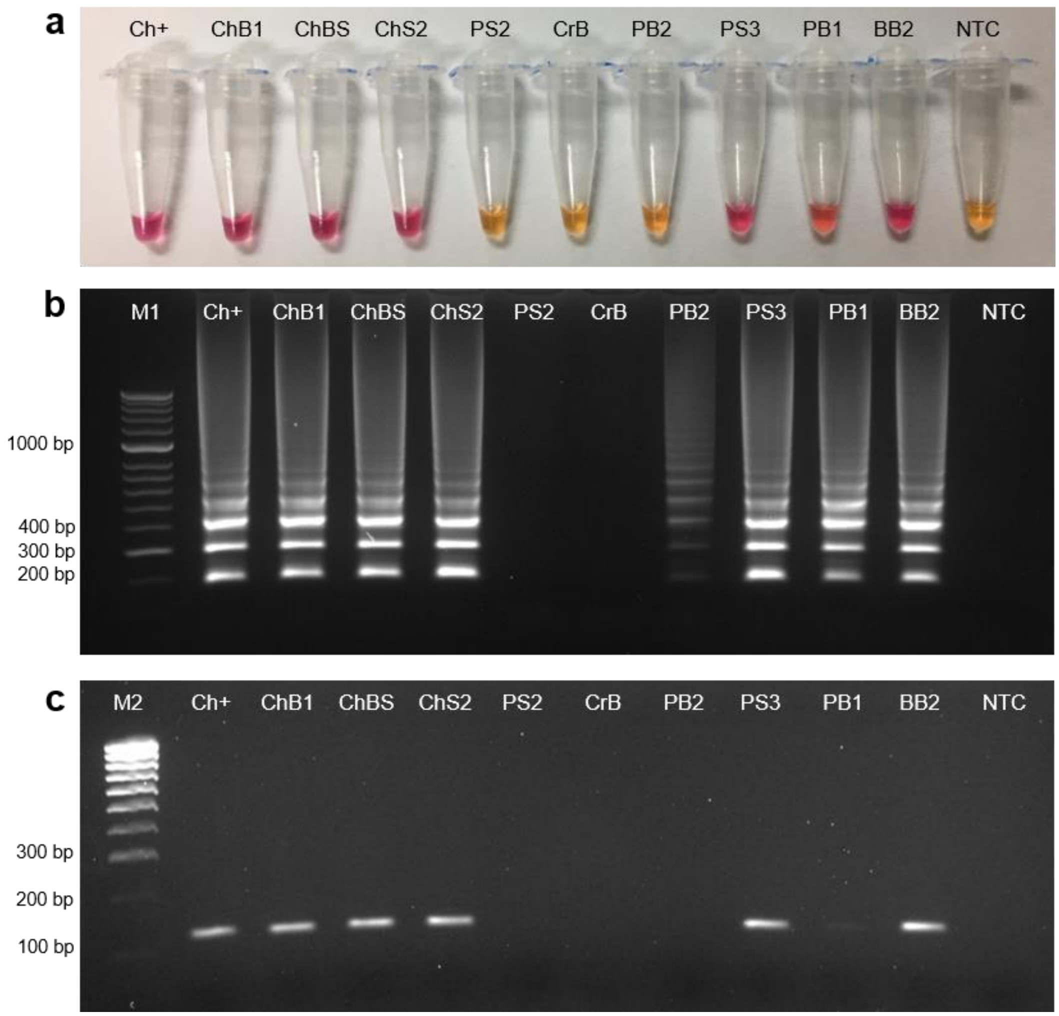

Fig. 5 Colourimetric LAMP and conventional PCR assays tested with commercial processed meat products. (a), the change of reaction colour after performing LAMP assay; (b), LAMP DNA amplicons from 1.5% (w/v) agarose gel electrophoresis and (c), as a comparison, conventional PCR was used as a gold standard to detect the presence of chicken DNA, PCR products were analysed using 2.0% (w/v) agarose gel electrophoresis. In this figure, some of the commercial products from Table 3 were chosen as representatives, which included (ChB1), sample no.1 - chicken ball-1; (ChBS), sample no.4 – chicken Bolognese sausage; (ChS2), sample no.7 – chicken sausage-2; (PS2), sample no.16 – pork sausage-2; (CrB), sample no.25 – crocodile meatball; (PB2), sample no.20 – pork ball-2; (PS3), sample no.17 – pork sausage-3; (PB1), sample no.19 - pork ball-1; and (BB2), sample no.22 – beef ball-2. (M1), HyperLadder 50 bp DNA marker (Bioline, UK); (M2), HyperLadder 100 bp DNA marker (Bioline, UK); (Ch+), pCR-Cytb-Chick plasmid as a positive control; and (NTC), deionised H_2O as a no template control.

Table 3 The detection results of colourimetric LAMP assays with commercial processed meat products. All the samples were tested with colourimetric LAMP assay and conventional PCR.

Sample No.	**Type of Sample**	**%meat declared on label**	**Detection result**	
			Colourimetric LAMP assay	PCR targeting Cytb gene
1	Chicken ball – 1	50% chicken	+	+
2	Chicken ball – 2	69.7% chicken	+	+
3	Chicken ball – 3	60% chicken	+	+
4	Chicken Bologna sausage	81% chicken	+	+
5	Chicken Vietnamese sausage	75% chicken	+	+
6	Chicken sausage – 1	53% chicken	+	+
7	Chicken sausage – 2	70% chicken	+	+
8	Chicken sausage – 3	70% chicken	+	+
9	Chicken sausage – 4	65% chicken	+	+
10	Chicken sausage – 5	65% chicken	+	+
11	Chicken sausage – 6	65% chicken	+	+
12	Chicken burger	77.76% chicken	+	+
13	Chicken nugget	43% chicken	+	+
14	Chicken roll	70% chicken	+	+
15	Pork sausage – 1	87% pork	ND	ND
16	Pork sausage – 2	87% pork	ND	ND
17	Pork sausage – 3	74% pork	+	+
18	Pork sausage - 4	74% pork	+	+
19	Pork ball - 1	80% pork	+	+
20	Pork ball – 2	80% pork	+	ND
21	Beef ball – 1	80% beef	+	ND
22	Beef ball – 2	95% beef	+	+
23	Beef burger	100% beef	ND	ND
24	Shrimp dumpling	35% shrimp	+	+

25	Crocodile meatball	99% crocodile	ND	ND
26	Dried crocodile meat – 1	100% crocodile	ND	ND
27	Dried crocodile meat – 2	92.5% crocodile	ND	ND
28	Dried crocodile meat - 3	100% crocodile	ND	ND

+, positive detection; ND, not detected.

3.5 Discussion: applicability of the assay and its limitations

In this work, a new analytical method based on colourimetric LAMP for authentication of chicken has been successfully developed. Our LAMP technique can be completely performed in one hour or less (Fig. 1d and 1e) without the need of additional detection steps as the change of pH-sensitive indicator colour can be observed immediately after the termination of LAMP. The colourimetric LAMP assay also offers high specificity to chicken DNA with no cross reactivity with other meat species, especially some close-related species such as turkey, duck and quail (Fig. 2a and 2b). Furthermore, our LAMP assay has been proven for its high sensitivity with the LOD for chicken DNA as low as 1 pg (Fig. 3a and 3b) and the LOD for chicken in binary meat admixtures of 0.01% (w/w) (Fig. 4a – 4d).

The detection of chicken content in raw meat materials or processed meat products based on PCR-based techniques including primer-specific PCR, multiplex PCR and nested PCR has been widely reported (Fujimura et al., 2008; Kim et al., 2018; Mane et al., 2009; Martín et al., 2007; Unajak et al., 2010). These methods are specific and sensitive for detection of chicken. However, the requirements for sophisticated machines like thermal cyclers for DNA amplification and the preparation of gel electrophoresis limit the practical use of the methods for field works and laboratories with limited resources (Ali et al., 2012). Real-time PCR (RT-PCR), another PCR-based method, has also been used to detect chicken DNA (Kesmen et al., 2012; Kim & Kim, 2019; Tanabe et al., 2007) with high specificity and sensitivity, yet skips analysis using gel electrophoresis (Farag et al., 2015). Again, RT-PCR still needs a special thermal cycle to perform the assay, making it unsuitable for resource-limited laboratory settings and on-site applications (Ali et al., 2012). Therefore, the development of a new bioanalytical assay based on LAMP could be a promising solution as the LAMP can be performed at a constant temperature using simple lab devices such as water baths and heating blocks, which are routinely affordable. LAMP assays combined with several techniques have been reported to achieve high sensitivity and specificity for chicken detection (Ahmed et al., 2010; Cho et al., 2014; Sul et al., 2019; Wang et al., 2020). In particular, the use of LAMP combined with lateral flow dipstick (Wang et al., 2020) is probably appropriate to be employed in the field works as it is easy to operate and portable, and the outcome can be detected by naked eyes. Our colourimetric LAMP assay has been made to be even more convenient and easier for detecting chicken in either raw matrices or processed meat products as the detection can be completely conducted in one step without the preparation of gel electrophoresis or the steps of reagent additions. The change of the reaction colour indicating positive or negative outcome can immediately be monitored after the LAMP incubation finishes. This is beneficial as there is no need for any additional steps, avoiding contamination from opening the reaction tubes. The main limitations of our method are that it can only deliver qualitative information and the sensitivity is not as high as RT-PCR (Table 2). However, with its affordable price, high sensitivity and specificity, ease of operation and portability, our colourimetric LAMP assay is suitable for on-site applications and low-resource laboratory settings.

4. Conclusion

In this work, a new detection method based on colourimetric loop-mediated isothermal amplification has successfully been developed for authenticating chicken in both raw meat materials and processed meat products. The assay has been proven for its high specificity to chicken without cross-reactivity with the other meat species tested. The sensitivity of the assay is high with the LOD for chicken DNA as low as 1 pg and the LOD for chicken content in binary meat admixtures of 0.01% (w/w). The colourimetric LAMP assay presented herein can also offer fast-processing time, ease of operation, and cost effectiveness since the assay only requires a simple thermostatic device. The outcome of the assay can easily be monitored from the change of the pH-sensitive indicator added, avoiding the possibility of contamination from opening the reaction tubes. It is thus expected that our colourimetric LAMP method could be applicable for laboratories with limited resources and on-site services, facilitating the accurate authentication of chicken in food industry.

Declaration of competing interest

The authors declare that there is no conflict of interest.

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