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RESEARCH ARTICLE
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Detection of Dog (*Canis lupus familiaris*) and Pig (*Sus scrofa*) Meat Components for Halal Authentication using Duplex Loop-Mediated Isothermal Amplification (Du-LAMP)

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ABSTRACT

This study aimed to develop and validate a duplex loop-mediated isothermal amplification (Du-LAMP) assay for the early, simultaneous detection of dog (*Canis lupus familiaris*) and pig (*Sus scrofa*) DNA in food products to support Halal authentication efforts. Species-specific primers targeting mitochondrial DNA were designed for both species. The assay was tested on raw and cooked meat samples under laboratory conditions. Specificity, sensitivity, and time-to-result were evaluated and compared against conventional PCR. Negative controls included Diethyl pyrocarbonate (DEPC)-treated water and non-target species. The Du-LAMP assay specifically detected both dog and pig DNA, with no cross-reactivity. The limit of detection was 200 femtograms, approximately 50 times more sensitive than conventional PCR. Amplification was completed within 30min at 68°C, with visualization by agarose gel electrophoresis. In contrast, PCR required ~90min and thermal cycling. This study reports the first duplex LAMP assay capable of simultaneously detecting dog and pig DNA in a single reaction. The method is rapid, sensitive, and potentially adaptable for field use in Halal compliance screening. Further validation on commercial food products is recommended to support real-world applications.

Keywords: Food authentication; Meat adulteration; Mitochondrial DNA; Molecular diagnostics; Species identification.

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INTRODUCTION

Consuming halal food is a religious obligation for nearly 2 billion Muslims worldwide and is one of the fastest-growing global markets, currently valued at over USD 580 billion, a figure expected to increase in the coming decades (Ahmad et al., 2025). This rising demand has intensified concerns about food integrity, particularly the risk of undeclared, adulterated, or substituted ingredients within complex supply chains (Usman et al., 2024; Naqvi et al., 2025). Among these, the presence of non-halal meat components—especially pork (*Sus scrofa*) and dog (*Canis lupus familiaris*)—poses serious challenges for religious compliance, consumer rights, and public health.

Conventional detection methods, such as electrophoresis or dielectric assessment, are often insufficiently sensitive and specific to identify traces of non-halal components in processed or cooked foods (Usman et al., 2024). In recent years, advances in testing technologies such as PCR, immunology, chromatography, spectroscopy, and new approaches such as CRISPR-Cas systems, artificial intelligence (AI), and stable isotope analysis have significantly improved the reliability of food authentication (Chaudhary & Kumar, 2022; Doroudian et al., 2024; Ellahi et al., 2025; Feng et al., 2024; Minoudi et al., 2025). At the same time, the global halal certification framework remains fragmented. Certification bodies such as JAKIM (Malaysia), MUIS (Singapore), MUI (Indonesia), GSO (Gulf Region), and SMIIC (OIC) apply different standards for slaughter procedures, raw material eligibility, and tracking systems. This lack of harmonization, coupled with high-profile cases of fraud involving unreported non-halal meat, has eroded consumer confidence and highlighted the urgent need for reliable, rapid, and cost-effective detection tools (Haji et al., 2023).

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To date, loop-mediated isothermal amplification (LAMP) assays have been developed for the detection of individual non-halal species, most commonly pig, *Sus scrofa* (Kanchanaphum et al., 2014; Thangsunan et al., 2021; Fang et al., 2025; Wang et al., 2025) and, less frequently, dog, i.e., Raccoon dog, *Nyctereutes procyonoides* (Liu et al., 2017). However, no existing study has reported a duplex LAMP (Du-LAMP) assay capable of simultaneously detecting both species, i.e., dog (*Canis lupus familiaris*) and pig (*Sus scrofa*), in a single reaction. This represents a critical gap in halal authentication, where screening for multiple non-compliant species in a single test is often necessary.

In this study, we developed and validated a duplex LAMP (Du-LAMP) assay to simultaneously detect pig and dog DNA in raw and cooked meat samples. The assay employs species-specific mitochondrial DNA primers and enables target differentiation via agarose gel electrophoresis. To our knowledge, this is the first duplex LAMP method tailored for halal authentication, allowing detection of two prohibited species in a single-tube reaction. This approach improves testing efficiency, reduces cost, and enhances the reliability of routine halal compliance screening.

MATERIALS & METHODS

Meat samples included dog, pork, beef, chicken, and rat, purchased from specialized traders operating in separate locations in Mataram City, West Nusa Tenggara, Indonesia. To prevent interspecies contamination, all meats were processed separately, stored at -20°C in the Immunobiology Laboratory of the University of Mataram, and handled using sterile techniques. Pre-processing steps included washing, slicing, and shredding prior to DNA extraction.

DNA Extractions

Genomic DNA was extracted from both raw and heat-treated (boiled at 100°C for 15min) dog and pig meat samples. Approximately 1g of each sample was finely chopped with a sterile scalpel, homogenized in 3mL phosphate-buffered saline (PBS; pH 7.4) using a mortar and pestle, and centrifuged at 1,000 × g for 10min at 4°C to remove tissue debris. The resulting supernatant ("meat extract") was used for DNA extraction.

For this study, genomic DNA was extracted from all five species to support the development and specificity testing of the duplex LAMP assay. Specifically, dog (n=3) and pig (n=3) samples were analyzed in both raw and heat-treated forms. In contrast, beef (n=3), chicken (n=3), and rat (n=3) samples were used exclusively for cross-reactivity evaluation.

DNA was extracted using the Geneius™ Micro gDNA Kit (GeneAid, Taiwan) according to the manufacturer's protocol. DNA concentration and purity were measured using a NanoDrop ND2000C spectrophotometer (Thermo Scientific). The A260/A280 ratios ranged from 1.6 to 1.8, indicating acceptable purity for downstream LAMP analysis. Extracted DNA was stored at −20°C until further use.

LAMP Primer Design

In this study, six LAMP primers (FIP, BIP, F3, B3, LF or LoopF, and LB or LoopB) were designed only for the two target species (dog and pig) using PrimerExplorer V5 (https://primerexplorer.eiken.co.jp/lampv5e/index.html, accessed 18 July 2024). Primers were not designed for beef, chicken, or rat, as these species served solely as non-target controls for duplex LAMP specificity evaluation.

Rather than analyzing the entire mitochondrial genomes of *Canis lupus familiaris* (Linnaeus, 1758) and *Sus scrofa* (Linnaeus, 1758), primers were developed from mitochondrial gene regions widely employed in species authentication due to their high interspecies variability, mitochondrial specificity, and abundant copy number (Kesmen et al., 2009; Ali et al., 2015; Farag et al., 2020; Zhang et al., 2020; Ibrahim et al., 2023; Depamede et al., 2025).

For *C. lupus familiaris* (dog), primers were designed to target conserved regions of the Cytochrome b (cyt b) gene (GenBank accession: AB048590.1) and the NADH dehydrogenase subunit 6 (ND6) gene. For *S. scrofa* (pig), primers were designed from the NADH dehydrogenase subunit 2 (ND2) gene (GenBank accession: AB292606.1). These loci were selected for their proven utility in molecular food authentication and species discrimination (Rastogi et al., 2007; Dawan & Ahn, 2022). All reference sequences were retrieved from GenBank (https://www.ncbi.nlm.nih.gov/genbank/, accessed 17 July 2024). All designed primers were evaluated for species specificity using NCBI BLAST (https://www.ncbi.nlm.nih.gov/, accessed 18 July 2024). The primers exhibited complete or partial identity with the intended mitochondrial targets (*C. lupus familiaris* and *S. scrofa*) and, in some cases, with non-target sequences, including Homo sapiens. No experimental cross-reactivity testing against non-target DNA was conducted in this study. To contextualize assay performance, primers previously published for dog and pig authentication (Widyanto et al., 2021; Tao et al., 2022) were also included. The final primer sets selected for use in this study are presented in Table 1 and 2. All primers were synthesized by Integrated DNA Technologies (IDT) (https://www.idtdna.com/pages/products/custom-dna-rna/dna-oligos).

LAMP Assay (Simplex and Duplex)

LAMP reactions were conducted in 20µLvolumes using species-specific primers targeting *Canis lupus familiaris* and *Sus scrofa* mitochondrial genes. Simplex-LAMP assays contained one species-specific primer set per reaction, while Duplex-LAMP combined both sets for simultaneous detection. Each Simplex reaction included 1.6µM inner primers (FIP/BIP), 0.2µM outer primers (F3/B3), 0.4µM loop primers (LoopF/LoopB), and 1ng of template DNA. In Duplex-LAMP, pig primers were maintained at Simplex concentrations, while dog outer primers were increased to 0.4µM to balance amplification efficiency based on preliminary optimization.

Table 1: LAMP primers for *Canis lupus familiaris* (dog) used in the present study

Primer	Sequence (5'–3')
F3-D1	GTAATCCAGGTCGGTTTCT
B3-D1	GTTTAAGTTTTACGCAGTTACC
FIP-D1	TAGTTTTAAGGCGCCTCTGTGAGCTATTATACAACCTCCCCCAG
BIP-D1	ATGAAGTCAACTCAATCTAACCAGTTCTGCACCCTAACAAAGC
LF-D1	CCTTACATCCCTTGTCCTTTCGTA
LB-D1	ATCTCCTCATAAGCCCGAGAAAAG
F3-D2	TGTTTACCAAAAACATCACCT
B3-D2	ATTGTGGTATTCCCGCCT
FIP-D2	CCTTTGCACGGTCAGGATACCGCATTTCTAGTATTGGAGGCA
BIP-D2	AGGGACTTGTATGAATGGCCAGGAAGGTCAATTTCACTGATTG
LF-D2	GCGGCCGTTAAACAAGTGTCA
LB-D2	CACGAGGGTTTAACTGTCTCTTACT

Primers are based on information from Depamede et al. (2025) with some modifications. Two sets were chosen due to their demonstrated capacity for strong and precise amplification in simplex reactions.

Table 2: LAMP primers for *Sus scrofa* (pig) used in the present study

Primer	Sequence (5'–3')
F3- P1	CACACGATAGCTAGGACC
B3-P1	CAAGGGTTGGTAAGGTCT
FIP-P1	ACTCTGGCGAATAGTTTTGTTATGTCTGGGATTAGATACCCCACTA
BIP-P1	ACTACTCGCAACTGCCTAAAACTTATCGATTATAGAACAGGCTCC
LF-P1	ACTATTTGGGTTTAGGGCTAGGC
LB-P1	TCAAAGGACTTGGCGGTGC

Primers are based on information from Depamede et al. (2025) with some modifications. P1 sets were chosen due to their demonstrated capacity for strong and precise amplification in simplex reactions.

Reactions were prepared in 1× Isothermal Amplification Buffer II (NEB) supplemented with 6mM $MgSO_4$, 14mM dNTPs, 0.2mM dUTP, 1M betaine, 0.5µL uracil-DNA glycosylase (UDG), 1.0µLBst 3.0 DNA polymerase (NEB), 1ng of mixed template DNA, and DEPC-treated water to volume. Reactions were incubated at 68°C for 30min, followed by enzyme inactivation at 98°C for 2min. Amplicons were separated on 2% agarose gels (1× TAE) stained with ethidium bromide (0.5µg/mL) and visualized using a GelDoc Fire-Reader (Uvitec, UK).

To minimize contamination, all LAMP preparation and analysis were performed in physically separated areas using dedicated pipettes, filter tips, and personal protective equipment. Work surfaces were decontaminated with RNase AWAY® (Sigma-Aldrich), and tubes were only opened after amplification in a designated post-reaction area.

PCR Amplification

Conventional PCR was performed in 20µL reactions containing 2× MyFi Mix (Bioline), 0.4µM F3/B3 primers, and the same DNA templates used in LAMP. Cycling conditions were initial denaturation at 94°C for 2min; 30 cycles of 94°C for 30sec, 59°C for 30sec, and 72°C for 30sec; followed by a final extension at 72°C for 5min. PCR products were analyzed by 2% agarose gel electrophoresis under the same imaging conditions as LAMP. DEPC-treated water served as the negative control.

RESULTS AND DISCUSSION

Design and Characteristics of LAMP Primers

The primary objective of this study was to detect dog and pork components in processed livestock-derived foods. Economic motives often drive the fraudulent inclusion of these meats in products such as meatballs and sausages, particularly in regions where they are non-halal or culturally sensitive (Ahmed et al., 2022; Haji et al., 2023; Ahmad et al., 2025). Because visual differentiation between dog, pork, beef, and chicken meat is nearly impossible after processing, molecular diagnostic tools are essential (Conter, 2024).

Simplex-LAMP reactions successfully amplified both dog and pork DNA using primers designed in this study. Agarose gel electrophoresis revealed the characteristic ladder-like banding pattern of LAMP products, confirming successful amplification (Fig. 1). Based on these results, a Duplex-LAMP assay was established using primer sets D2 for *Canis lupus familiaris* (Table 1) and P1 for *Sus scrofa* (Table 2). These sets were selected because they showed the most robust and specific amplification in simplex reactions. In silico BLAST analysis further confirmed minimal off-target binding.

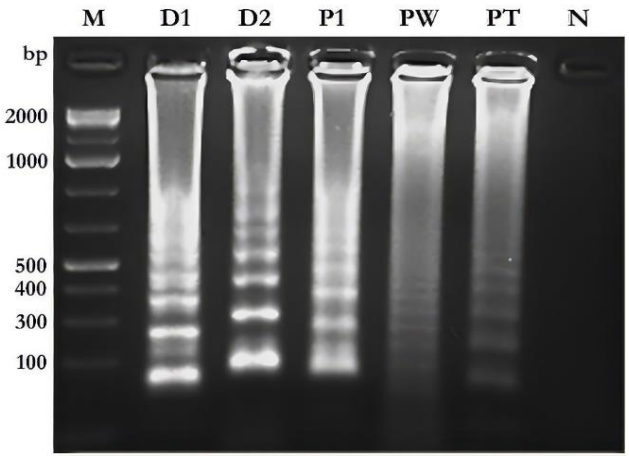

Fig. 1: Representation of agarose gel (2%) electrophoresis of simplex-LAMP products using different primer sets for dog and pork DNA. D1 and D2, primer sets for *Canis lupus familiaris* (dog); P1, PW, and PT, primer sets for *Sus scrofa* (pig/pork). All tested primer sets successfully amplified their respective targets, producing the characteristic ladder-like banding pattern of LAMP products. Among them, D2 (dog) and P1 (pork) showed the strongest and most specific amplification and were selected for subsequent Duplex-LAMP development (Table 1). M: 100bp DNA ladder (Himedia); N: no-template negative control, DEPC-treated water. (Experiments were repeated at least three times.)

Although a comprehensive quantitative comparison across all candidate primers (e.g., Ct values or melting curve analysis) was not performed, the strong empirical performance of D2 and P1 justified their use. This limitation is acknowledged, and future studies should apply real-time LAMP (qLAMP) or digital LAMP (dLAMP) to enable quantitative primer validation, including reaction kinetics and sensitivity thresholds as reported by others (Papadakis et al., 2022; Zhang et al., 2023; Hartle-Mougiou et al., 2024; He et al., 2025).

Simultaneous Reaction of Duplex-LAMP

Practical halal authentication tools must deliver speed, accuracy, specificity, and sensitivity. Here, we developed a duplex LAMP assay to simultaneously detect dog and pork DNA in processed meat products by combining species-specific primers in a single reaction. Optimal duplex amplification was achieved by maintaining the pig primer

concentrations from the Simplex assay and increasing the dog outer primers (F3 and B3) from 0.2 to 0.4μM. This adjustment significantly improved amplification performance (Fig. 2A). The optimized Duplex-LAMP reaction was conducted at 68°C for 30min.

Specificity was tested against DNA from beef, chicken, and rat under identical conditions. No amplification was observed in these non-target species (Fig. 2B), confirming the high specificity of the primer sets. These findings are consistent with earlier reports showing that optimized primer combinations can enhance LAMP assay performance (Wang et al., 2020; Yang et al., 2022; Zhuang et al., 2024). Based on these results, the optimized Duplex-LAMP formulation was adopted for subsequent experiments.

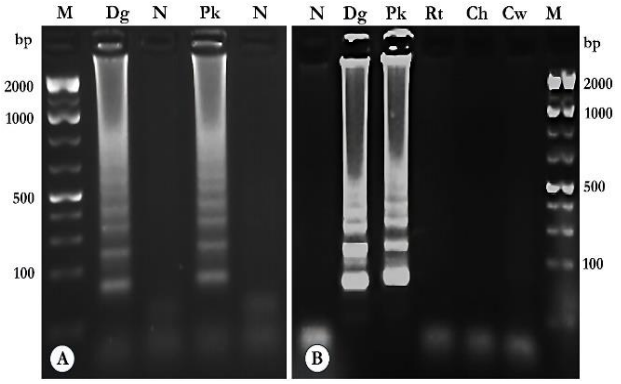

Fig. 2: Representations of agarose gel (2%) electrophoresis of Duplex-LAMP assay optimization and specificity testing. A) Optimization of primer concentrations for simultaneous detection of dog (Dg) and pork (Pk) DNA. Increasing the concentration of dog outer primers (F3/B3, 0.4μM) improved amplification performance, and B) Specificity testing of the optimized Duplex-LAMP assay using DNA from non-target species (Cw, beef; Ch, chicken; Rt, rat). No cross-amplification was observed. M: 100bp DNA ladder (Himedia); N: no-template negative control, DEPC-treated water (Experiments were repeated at least three times).

Reaction of Duplex-LAMP on Boiled Dog Meat and Boiled Pork

For practical applications, halal authentication must also be feasible on cooked products. Therefore, Duplex-LAMP was tested on DNA from both frozen and boiled (100°C for 15min) dog and pork meat samples. In parallel, PCR using dog- and pig-specific F3/B3 primers was performed on the same samples. Both Duplex-LAMP and PCR successfully detected DNA from frozen and boiled samples (Fig. 3), demonstrating that the developed Duplex-LAMP assay can be applied to cooked food matrices. While PCR produced comparable results, it required nearly three times as long as Duplex-LAMP (90 vs. 30min). These findings are consistent with prior reports highlighting the time-saving advantages of LAMP over conventional PCR and even RT-PCR (Soroka et al., 2021).

Sensitivity Testing of Duplex-LAMP Compared to PCR

Both Duplex-LAMP and PCR were further assessed for their ability to detect dog and pork DNA from boiled meat samples. Instead of standard 10-fold serial dilutions, DNA was diluted at broader intervals of 1-, 100-, 1000-, and 5000-fold from an initial concentration of 1ng/μL. For both assays, 1μL of each dilution was used in a 10μL reaction. Duplex-LAMP reactions were conducted at 68°C for 30min, while PCR was performed under the cycling conditions described previously.

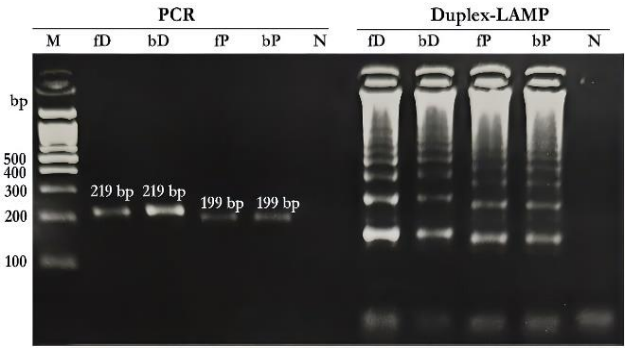

Fig. 3: Representation of agarose gel (2%) electrophoresis of PCR and Duplex-LAMP products from frozen and boiled meat samples. Duplex-LAMP successfully amplified DNA from both frozen and boiled samples, producing the characteristic ladder-like banding pattern of LAMP products (~150bp to >1000bp). In parallel, conventional PCR generated single, discrete amplicons of the expected sizes: 219bp for *Canis lupus familiaris* (D2 primers) and 199bp for *Sus scrofa* (P1 primers). Each reaction contained 10 ng of template DNA. fD, frozen dog meat; bD, boiled dog meat; fP, frozen pork; bP, boiled pork. M: 100bp DNA ladder (Himedia); N: no-template negative control, DEPC-treated water (Experiments were repeated at least three times).

Duplex-LAMP consistently detected DNA down to 200fg (5000-fold dilution), whereas PCR detection was limited to 10pg (100-fold dilution), indicating ~50-fold higher sensitivity of Duplex-LAMP (Fig. 4A and 4B). Although highly sensitive, the exact LOD was not established; finer 10-fold dilutions would be required for precise determination. Resource constraints prevented such testing in this study, but future work will incorporate real-time LAMP formats for quantitative LOD refinement and kinetic analysis. Previous studies have reported even lower LODs (1–10 fg) for LAMP assays (Mori and Notomi, 2009; Notomi, 2000; Sadeghi et al., 2021). Such variability likely reflects differences in primer design, reaction conditions, and the presence of inhibitors in food matrices. Primer efficiency plays a central role in amplification, while substances in processed foods may interfere with enzyme activity and reduce sensitivity (Sheu et al., 2018; Yang et al., 2022). Despite the inherent challenges of multiplex reactions—including the risk of primer-dimer formation and reduced efficiency—the Duplex-LAMP assay outperformed conventional PCR in terms of sensitivity (Zanoli and Spoto, 2012). Further optimization using qLAMP or dLAMP could enhance quantification and reproducibility by providing threshold time (Ct) data and amplification kinetics.

Although LAMP has been widely validated for pathogen detection and food safety (Yang et al., 2018; Lakshmi & Kim, 2021; Ahmad et al., 2025), its application in halal authentication remains limited. Most published studies emphasize primer design and laboratory sensitivity testing, with few addressing field-relevant challenges such as cross-contamination, matrix complexity, or DNA degradation (Kim & Kim, 2018; Ng et al., 2022). To address this gap, we evaluated Duplex-LAMP's performance under simulated field conditions. No cross-reactivity was

observed with beef, chicken, or rat DNA (Fig. 2B), and the assay reliably detected DNA from heat-treated samples, confirming both specificity and resilience to thermal degradation. Despite these promising results, the lack of standardized protocols for LAMP-based halal testing remains a barrier to adoption. Variables such as DNA extraction, sample preparation, and detection format (e.g., colorimetric, turbidimetric, or lateral-flow readouts) must be optimized to minimize false results (Kim et al., 2023; Fathima et al., 2024; Yang et al., 2024).

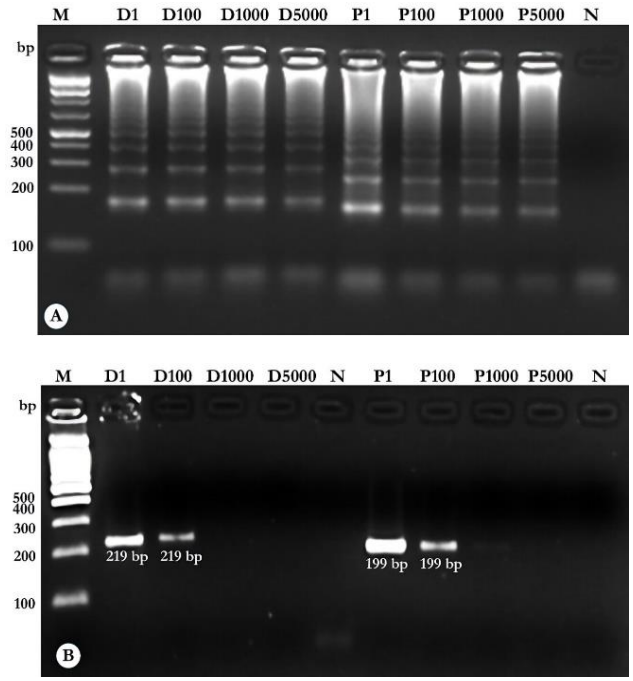

Fig. 4: Representation of agarose gel (2%) electrophoresis for sensitivity comparison of Duplex-LAMP and PCR assays using diluted DNA from boiled dog (A) and pork (B) meat samples. (A) Duplex-LAMP assay performed at 68°C for 30min. (B) PCR assay performed under the cycling conditions described in Methods. For both assays, DNA templates were diluted 1-, 100-, 1000-, and 5000-fold from an initial concentration of 1ng/µL. D1–D5000 and P1–P5000 represent dilutions of dog and pork DNA, respectively. Each reaction used 1µL of diluted DNA in a 10µL total volume. M, 100bp DNA ladder (Himedia); N, negative control (DEPC-treated water). Duplex-LAMP consistently detected DNA down to 200fg (5000-fold dilution), whereas PCR detection was limited to 10pg (100-fold dilution), demonstrating ~50-fold higher sensitivity of Duplex-LAMP (Results of twice repeated experiments).

In summary, Duplex-LAMP demonstrated high sensitivity and specificity but requires further validation under real-world conditions. Future studies should investigate the effects of food matrix complexity, additives, preservatives, and sample handling. Integration with portable detection platforms could further support on-site testing and facilitate uptake by halal certification authorities. Bridging these gaps will be crucial for consumer trust and real-world applications. The Duplex-LAMP assay presented here provides a rapid, cost-effective, and sensitive tool for the simultaneous detection of *Canis lupus familiaris* and *Sus scrofa* DNA, advancing molecular halal authentication.

Conclusion

This study reports the successful development of a duplex LAMP (Du-LAMP) assay for the simultaneous detection of dog and pork meat components. The method enables sensitive detection of both raw and cooked meat, reaching femtogram-level DNA concentrations, and delivers results within ~30min at 68°C. These features highlight Du-LAMP as a rapid and cost-effective tool with strong potential for use in halal authentication laboratories and compliance centers. Future research should focus on validating assay performance under real-world conditions and assessing its scalability for routine industrial and field applications.

DECLARATIONS

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Conflict of Interest: All authors have read and approved the final version of the manuscript and declare no conflict of interest.

Data Availability: All the data generated during the study are present in the article.

Ethics Statement: Ethical approval was not required for this study. DNA was extracted from animal meat samples obtained from laboratory stocks or purchased from local markets. All animals were slaughtered in compliance with relevant regulations and animal welfare standards for Indonesia (Pemerintah Indonesia, 2012).

Author's Contribution: Conceptualization, investigation, methodology, primer development, supervision: S.N.D.; data curation and analysis, manuscript review: M.S.; preparation of halal biological materials, manuscript review: Dj.K.

Generative AI Statement: The authors declare that no Gen AI/DeepSeek was used in the writing/creation of this manuscript.

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