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Detection of Porcine Contamination in Beef Meatballs from Gedangan District Using PCR–RFLP

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Abstract. *Meatball is one of the popular meat dishes in Indonesia, especially in Sidoarjo. The sale of meatballs in Sidoarjo is carried out by Micro, Small, and Medium Enterprises (MSMEs), with 330 of the total 11,114 micro-traders selling meatballs. However, there is a risk of pork being adulterated into meatballs. This is a serious problem because Indonesia, especially Sidoarjo, is a Muslim-majority city with 95.5% of the total population of 2 million. Therefore, the purpose of this article is to conduct field checks to determine whether or not pork contamination is present in meatballs in Sidoarjo. Sampling locations were determined using a stratified random sampling method, which found that eight of the 15 villages, including Karangbong, Tebel, Sruni, Keboananom, Keboansikep, Gedangan, Ketajen, and Sawotratap Villages, have a significance value greater than the alpha value (0.05). Therefore, eight of them were used as sampling locations. One sample was purchased from each of the eight chosen villages, named S1, S2, S3, S4, S5, S6, S7, and S8. Meanwhile, the possibility of porcine contamination in beef meatball samples was analyzed using the Polymerase Chain Reaction- Restriction Fragment Length Polymorphism (PCR-RFLP) method with the restriction enzyme BsaJI. This study found that all eight samples and the negative control DNA amplicons were not cleaved by the BsaJI enzyme. Meanwhile, the positive control DNA amplicon was cleaved into 3 fragments. It was indicated that all samples and the negative control DNA amplicons were free of pork DNA contamination. Despite the anomaly, it can be concluded that all meatball samples bought from Gedangan District are not contaminated with pork. By demonstrating that meatballs from Sidoarjo are free from pork DNA, this study serves as an additional halal food monitoring in Sidoarjo. It reinforces public trust in local MSME food products.*

Keywords*: BsaJI, detection, food, halal, meatball, PCR-RFLP*

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Jurnal Biodjati 10(2): 332–351, November 2025

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INTRODUCTION

Meatballs are a popular food that can be easily found in Sidoarjo. The sale of meatballs in Sidoarjo is carried out by Micro, Small, and Medium Enterprises (MSMEs). Based on data from the Sidoarjo Regency Cooperative and Micro Business Office in 2025, there were 330 micro-enterprise meatball sellers (Ditakopum, 2025). Meatball vendors registered as business entities account for 2.97% of the total 11,114 food business entities registered in Sidoarjo Regency.

Meatballs are made from various meats, including beef and chicken. In Sidoarjo Regency, the raw material for beef meatballs costs about IDR 110,000 per kg, whereas for chicken meatballs, it is around IDR 35,000 per kg (SISKAPERBAPO, 2024). The price gap may incentivize economically motivated adulteration by substituting cheaper meats, such as pork, which is sold online for IDR 60,000. This is a potentially serious problem because Indonesia is a country with Islam as the religion with the largest believers. In 2022, the religion had over 241.7 million believers, or about 86.88% of Indonesia's population (Kementerian Agama Republik Indonesia, 2024). Moreover, Sidoarjo is also a Muslim-majority city with 95.5% of the total population of 2 million (BPS, 2024). Therefore, preventing non-halal species in widely consumed products such as meatballs is essential to guarantee halal food (Syafrida, 2016). Consequently, beef meatballs constitute a critical food matrix for halal testing in Sidoarjo. Accordingly, we analyzed beef meatballs sold in Gedangan District, Sidoarjo Regency.

Gedangan, a central urban district on Sidoarjo's airport-commercial corridor, hosts 3,147 MSMEs or 28.32% of the total registered MSMEs in Sidoarjo Regency, thereby providing a practical setting for complementary local halal surveillance (BPS Statistics Sidoarjo Regency, 2024). Relative to factory-packaged processed meats, e.g., sausages and nuggets, that are produced in licensed facilities and must hold BPOM processed-food labels/registration numbers with ingredient disclosure and batch certification, the beef meatballs sold by MSMEs and street vendors represent a higher-priority matrix for halal monitoring in the local retail environment (BPOM, 2018).

The government contributes to guaranteeing halal food and food safety for the people. The government stipulates that all food and beverage products, including those sold by street vendors and roadside stalls, must be halal-certified. The Halal Product Guarantee Law (Law of the Republic of Indonesia No. 33 of 2014 on Halal Product Assurance) and its implementation (Government Regulation of the Republic of Indonesia No. 39 of 2021 on Implementation in the Field of Halal Product Assurance) regulate the obligation of halal certification for all business scales. As an implementer at the local level, the Sidoarjo Regent issued a Circular Letter (Government of Sidoarjo Regency, 2025), requiring all culinary businesses in his area to immediately apply for a halal certificate through the Halal Product Guarantee Agency (BPJPH). However, scheduled supervision for street vendors or roadside stalls has not been systematically carried out and is rarely published. To our knowledge, no publicly documented case of pork adulteration in meat products has been reported in Gedangan District as of October 2025. However, Sidoarjo Regency previously recorded incidents in 2016 of pork or wild boar misrepresented by sellers as beef in traditional markets, e.g., Pasar Wadung Sari, Waru (Abidin, 2016). Moreover, local market oversight remains a concern; in 2025, 88% of sampled meats in five Sidoarjo markets, including Gedangan, were classified as 'gelonggongan' or water-injected beef (Rohman, 2025). Therefore, this study was conducted to analyze Gedangan meatballs and to serve as an additional monitoring of food halal at the local level (Gedangan).

Pork contamination in foods can be analyzed using PCR-RFLP (polymerase chain

reaction-restriction fragment length polymorphism). The PCR method is a molecular process that enzymatically amplifies multiple copies of a selected DNA region (Artika et al., 2022). Then, the RFLP method is conducted to analyze genetic variety by using restriction enzymes to cleave species-specific sites, producing diagnostic fragment patterns (Rani et al., 2024). The PCR-RFLP method was performed using the cytochrome b primer, which yielded a 359 bp amplicon, and BsaJI restriction enzyme (recognition site CCNNGG), which cleaved the pork cytochrome b amplicon. In contrast, the beef amplicon remained uncut (Adiningsih et al., 2018). Compared with the PCR method, PCR-RFLP adds a second specificity filter beyond primer binding and reduces false positives from non-specific amplification. Meanwhile, compared with RFLP without PCR, PCR-RFLP is far more sensitive for degraded DNA typical of processed foods (Siswara et al., 2022). Moreover, prior work using BseDI (homolog with BsaJI restriction enzyme) on cytochrome b amplicon could reliably detect 1% pork adulteration in meatball mixtures and differentiate pork from beef/chicken (Erwanto et al., 2013).

Research on pork contamination has been conducted in various cities, including Padang City. Irwan, et al., (2020) studied the pork contamination in the meatball in Padang City using PCR, and found 2 of 3 beef meatball samples had been contaminated by pork with 100% fragment similarity to Sus scrofa DNA fragments. In the Yogyakarta and Surabaya regions, Erwanto et al. (2014) used PCR-RFLP techniques. They found that 9 of 20 meatball shops in Yogyakarta were contaminated with pork, whereas 19 of 20 meatball shops in the Surabaya region were not. There is also research on pork contamination detection, which found that 22 of 36 commercial beef meatballs in Boyolali Regency contained pork, whereas none were detected in 36 samples in Bojonegoro Regency (Siswara et al., 2022).

Among the previous research, no scientific reports have been found regarding the identification of pork contamination or the halal status of beef meatballs specifically in the Gedangan District. Furthermore, there is a lack of published scientific research addressing the halal integrity of meatball products in this area. Therefore, this study is essential for providing initial scientific evidence and monitoring of potential pork contamination in beef meatballs circulating in Gedangan District.

MATERIALS AND METHODS

This research was carried out in April – May 2024 in the PT Pawitra Jaya Sakti Biotek for DNA isolation, visualization using electrophoresis, purification, and RFLP; while the DNA quantification was conducted in Laboratory of Molecular Biology, Faculty of Mathematics and Natural Sciences, State University of Surabaya (Unesa).

Instrument

Instrument used in this study are: total DNA Isolation used analytical balance (BIOBASE BP12002), microtube (BIOBASE), vortex (BIOBASE BK-VX1), centrifuge (BIOBASE D2012Plus), and micropipettes (BIOBASE); sample storing used 4°C refrigerator (BIOBASE BPR-5V650) and -20°C refrigerator (BIOBASE BDF-25V350); DNA quality used Nanodrop (Thermo SCIENTIFIC Nanodrop 2000 spectrophotometer); electrophoresis used hotplate magnetic stirrer (BIOBASE BK-MS280) for agarose making, analytical balance (BIOBASE BP12002), electrophoresis instruments (BIOBASE) to run the assay, and transilluminator (VISION MED Blue LED Transilluminator TI-470) to visualized the band after running the assay; Polymerase Chain Reaction (PCR) used PCR machine (VISION MED Gradient Thermal Cycler IG-96G) and micropipettes (BIOBASE); amplicon purification used MEBEP kit for purification from agarose; and Restriction Fragment Length Polymorphism (RFLP) used waterbath (BIOBASE WT-42), micropipettes (BIOBASE), and microtube (BIOBASE).

Jurnal Biodjati 10(2): 332–351, November 2025

JURNAL BIODJATI

http://journal.uinsgd.ac.id/index.php/biodjati

Materials

The materials used in the study consisted of beef meatballs from Karangbong, Tebel, Sruni, Keboananom, Keboansikep, Gedangan, Ketajen, and Sawotratap Villages, food DNA isolation kit (Macherey-Nagel NucleoSpin genomic DNA from food kit), Loading Dye (Promega Blue/Orange 6X Loading Dye), DNA marker 2000 bp (Tinzyme DL2000+ DNA Marker DM01A), DNA marker 100 bp (Tinzyme 100 bp DNA Marker DM10), 70% isopropyl alcohol, isoamyl alkohol, agarose, Gel Extraction kit (Mebep Bioscience DRK0101), GoTaq Green Master Mix (Promega), BsaJI enzyme (Thermo Scientific™), Nuclease Free Water, and universal cytochrome b forward and reverse primer (Integrated DNA Technologies).

Determination of Sampling Location

This study used Stratified Random Sampling (SRS) as the sampling method. The calculation of region significancy using stratified random sampling which is random sample collection technique from each homogenous strata (Sumargo, 2020) using following formula:

$$sample = \left(\frac{population\ size\ in\ a\ strata}{total\ population}\right) \times total\ sample\ size$$

From the results of the stratified random sampling calculation, the proportional value will be obtained. Villages with proportional value ≥0.5 were considered statistically indistinguishable from the district profile and thus eligible as strata (Han et al., 2024). Eight villages met this criterion and were included as sampling strata; from each eligible village, one vendor was randomly selected and one serving of cooked beef meatballs was purchased, yielding eight samples (n=8). Villages with proportional value <0.5 were not sampled to avoid bias toward atypical subpopulations. The villages were showed in Figure 1.

Sample collection

Sampling was done in April 2024 by buying cooked meatball samples directly from the random seller from each eight beef

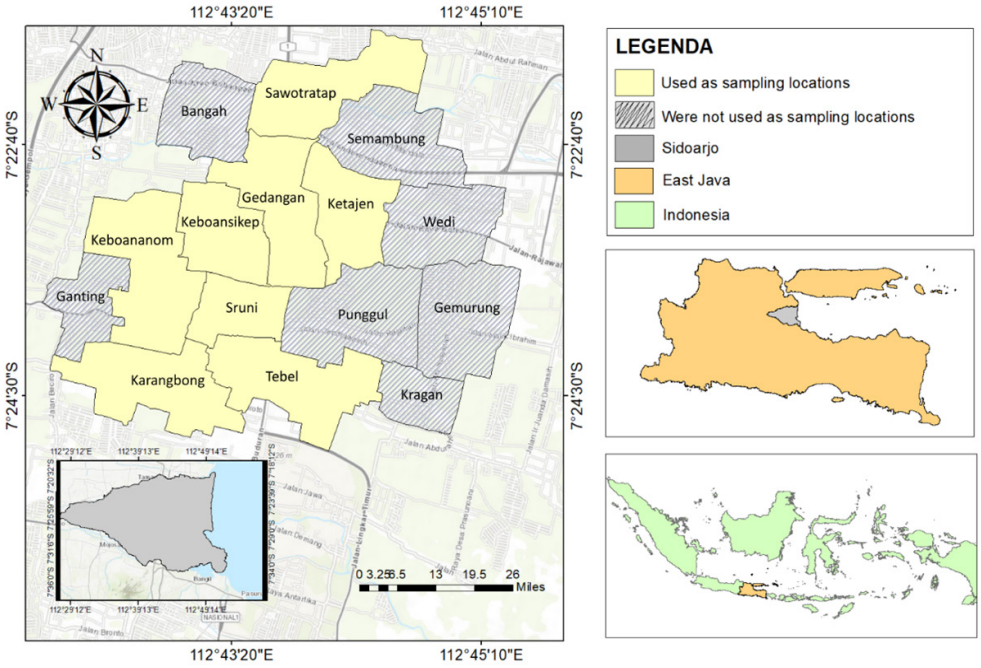

Figure 1. Sampling location; Karangbong = S1; Tebel = S2; Sruni = S3; Keboananom = S4; Keboansikep = S5; Gedangan = S6; Ketajen = S7; and Sawotratap = S8.

meatballs from Karangbong, Tebel, Sruni, Keboananom, Keboansikep, Gedangan, Ketajen, and Sawotratap Villages. The samples were stored in polypropylene bags and transported to the laboratory in the PT Pawitra Jaya Sakti Biotek. After that, 3 meatballs per sample will be taken and put into a new polypropylene bag that has been labeled. The samples in these new bags will be stored in a -20°C refrigerator until used in the next test.

Total DNA Isolation

DNA isolation was carried out in in April 2024 according to the manual of food DNA isolation kit (Macherey-Nagel NucleoSpin genomic DNA from food kit), which follows 8 steps, first the samples were homogenized using laboratory mortar and pestle, which there were 10 samples (200 mg for each sample), consisted of 8 meatball samples (one each from Karangbong, Tebel, Sruni, Keboananom, Keboansikep, Gedangan, Ketajen, and Sawotratap Villages), 1 positive control (minced pork), and 1 negative controls (minced beef); Second, the cells were lysed by adding 10 µL Proteinase K in 65 °C for 30 minutes, then centrifuged at 10,000×g for 10 min, and took 1 volume of the supernatant; third the DNA binding condition were adjusted by adding 1 volume C4 buffer and 1 volume absolute ethanol; forth the DNA binded by adding 750 µL into NucleoSpin tube then centrifuged at 11,000 ×g for 10 min; fifth the silica were washed and dried by first wash in 400 µL CQW buffer and centrifuged for 11,000×g in 1 min, second wash in 700 µL C5 buffer and centrifuged for 11,000×g in 1 min, then third wash in 200 µL C5 buffer and centrifuged at 11,000×g for 2 min. Finally, the DNA was eluted by adding 100 µL pre-heated CE buffer, then incubated at room temperature for 5 min and lastly centrifuged at 11,000×g for 1 min. Then, total isolated DNA was visualized using 1% agarose electrophoresis and quantified using NanoDrop with CE buffer as a blank.

Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is performed to amplify a mitochondrial cytochrome-b fragment for species authentication because mitochondrial targets provide a high copy number and remain detectable in processed foods (Siswara et al., 2022). The reaction mixture and the program for DNA amplification using PCR are listed in Table 2 and Table 3, respectively, and the oligonucleotide universal primer for cytochrome b was ordered from Integrated DNA Technologies with sequences shown in Table 1. Cytochrome b universal primer was used to amplify a 359 bp mitochondrial cytochrome b fragment because it is widely used for vertebrate species identification in recent work (Khan et al., 2025). Universal mitochondrial DNA primers are also recommended for degraded/processed matrices because mitochondrial DNA is present at high copy number and universal priming sites maintain amplification success when DNA is fragmented (Jung et al., 2024).

Meanwhile, PCR optimization is performed to enhance sensitivity, specificity, and stability when standard reaction conditions produce non-specific products, weak signals,

Table 1. Cytochrome-b universal primer

Primer	Sequence	Nucleotide Length (nt)
Cytochrome b Forward	CCATCCAACATCTCAGCATGATGAAA	26
Cytochrome b Reverse	GCCCCTCAGAATGATATTTGTCCTCA	26

or smeared bands that compromise reliable detection (Sun, 2025). The PCR optimization was carried out with gradient PCR for annealing temperature with other programs using the same temperature and time as listed in Table 3. The annealing temperatures ranged from 60 °C to 64 °C, with each temperature divided into 12 columns, as shown in Table 4. Then, the next three programs with the best results were validated with PCR using the selected or the best sample as template DNA and compared with the positive control. The next step used optimized PCR with a similar composition listed in Table 2 and Table 3. Except that the annealing temperature was adjusted to the optimal temperature attained from PCR optimization.

Amplicon Purification

Amplicon purification is performed to select a specific band and remove nonspecific bands and primer dimers; therefore, the downstream reaction will be clearer (Abraham et al., 2017). The amplicon purification was

Table 2. Mixture of sample and reagent with 1× reaction (Fadhlurrahman *et al*., 2015)

Components	Volume (µL)
Go Taq Green Master Mix (Promega)	12.5
Primer forward	1
Primer reverse	1
DNA template	5
Nuclease free water	5.5
Total Volume	**25**

Table 3. PCR programs (Fadhlurrahman *et al*., 2015)

Program	Temperature (°C)	Time (second)	Cycle
Predenaturation	94	120	1
Denaturation	94	36	35
Annealing	51	73	35
Extention	72	84	35
Final-extention	72	180	1

Table 4. Gradient PCR temperatures

Well	Code	Annealing temperature (°C)
1	G1	60.0
2	G2	60.3
3	G3	60.5
4	G4	60.9
5	G5	61.3
6	G6	61.8
7	G7	62.2
8	G8	62.7
9	G9	63.1
10	G10	63.5
11	G11	63.8
12	G12	64.0

Table 5. RFLP mixture using BsaJI to cleave DNA amplicon

Components	1× Reaction RFLP Volume (µL)	0.5× Reaction RFLP Volume (µL)
Purified amplicon sample	10	5
Nuclease free water	18	9
CutBuffer	1	0.5
Restriction enzyme BsaJI	1	0.5
Total Volume	**30**	**15**

performed according to MEBEP BIOSCIENCE Gel Extraction kit, which has 8 steps. The first step, the band excision, was conducted by illuminating the amplicon band under the long-wave ultraviolet light, then it was excised by a sterilized stainless steel spatula or cutter to get a smaller gel volume and amplicon ratio. Second, the weighing, the excised gel containing amplicon bands was put in a 1.5 mL centrifuge tube, which had been weighed, then the weights were subtracted to get the cut gel weights. Third, the dissolution buffer addition, buffer DD was added 3 times the volume of gel. If the gel weighs 100 mg, then its volume can be regarded as 100 µL; therefore, 300 µL Buffer DD shall be added. If the gel concentration is greater than 2%, the volume added shall be 6 times the gel volume. Fourth, the gel dissolution, the centrifuge tubes were incubated in a 56 °C water bath for 10 minutes, or until the gel was completely dissolved, and vortexed every 2-3 minutes to accelerate dissolution. Fifth, the column conditioning and the binding, new silicon membrane absorption columns, which were placed in collection tubes, were treated by adding 100 µL Balance Buffer, then centrifuged at 13,000 rpm for 1 min, and the flow-through was discarded. After absorption columns were treated, the solutions obtained from the previous step were added and incubated at room temperature for 1 min, then centrifuged at 12,000 rpm for 60 seconds, and the flow-through was discarded. Sixth, the wash I, 600 µL Buffer WB was added, then centrifuged at 12,000 rpm for 30 seconds, and the flow through was discarded. Seventh, the wash II and the dry spin, the sixth step was repeated, then put back again, and the tube was centrifuged at 12,000 rpm for 2 minutes to remove as much Buffer WB as possible. Eight, the elution, after putting the adsorption column in a new 1.5 mL centrifuge tube, the pre-heated Buffer EB (in 65-70°C temperature) was added to the middle of the adsorption membrane, then incubated at room temperature for 2 minutes and centrifuged at 12,000 rpm for 1 minute. And then the collected amplicons were visualized with 1% agarose electrophoresis.

Restriction Fragment Length Polymorphism (RFLP)

The Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR–RFLP) is an accurate, efficient, inexpensive, and simple-step method of SNPs exploration, validation, and characterization generated by using restriction enzymes. The restriction enzymes are enzymes that cut the DNA at specific sequences (Rosalinda et al., 2024). Halal test using PCR-RFLP on mitochondrial DNA marker, such as cytochrome b, continues to be a standard approach with recent works that use species diagnostic restriction patterns, including BsaJI on porcine cytochrome b with CCNNGG recognition site (Muflihah et al., 2023). The RFLP protocol was carried out using each of 10 µL purified amplicon. Then each amplicon will be mixed with BsaJI enzyme restriction 2 unit/µL for a volume of 0.5× reaction RFLP volume and incubated in a waterbath for 1 hour, with the overall reaction mixture displayed in Table 5.

Jurnal Biodjati 10(2): 332–351, November 2025

JURNAL BIODJATI

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Data analysis

Data were analyzed descriptively for gel-based visualizations (total DNA isolation, PCR, and RFLP) and quantitatively for DNA purity and concentration. DNA concentration (ng/µL) was obtained from Nanodrop (Thermo SCIENTIFIC Nanodrop 2000 spectrophotometer) absorbance at 260 nm for double-stranded DNA using: concentration =A260×50×dilution factor; purity was assessed from A260/A280 and A260/A230 ratios (Versmessen et al., 2024). The data collected were analyzed by using descriptive qualitative methods on the visualization for total DNA isolation results, PCR results, and RFLP results; and by using quantitative methods to analyze purity and concentration values of DNA isolates.

RESULTS AND DISCUSSION

Determination of Sampling Location

Gedangan District, the sampling location, was one of the districts in Sidoarjo Regency. It has a territory of 24.06 km2 with 15 villages, including Ganting, Karangbong, Tebel, Kragan, Gemurung, Punggul, Sruni, Keboananom, Keboansikep, Gedangan, Ketajen, Wedi, Semambung, Sawotratap, and Bangah (BPS Statistics Sidoarjo Regency, 2024). To determine which villages are eligible for use as sampling locations, stratified random sampling was used, with a threshold of 0.5 (Sumargo, 2020; Han et al., 2024). Stratified random sampling calculation results were shown in Figure 2. Among the 15 villages in Gedangan District, eight achieved a proportional value ≥0.5: Karangbong, Tebel, Sruni, Keboananom, Keboansikep, Gedangan, Ketajen, and Sawotratap. Eight of them are eligible to represent Gedangan District, and the samples purchased from them will be named S1, S2, S3, S4, S5, S6, S7, and S8, respectively. Meanwhile, Ganting, Kragan, Gemurung, Punggul, Wedi, Semambung, and Bangah Villages had a proportional value <0.5. Therefore, these villages were treated as atypical and excluded.

Total DNA isolation

Total DNA isolates from samples 1 to 8 were visualized with 1% agarose electrophoresis in Figure 3. Several lanes showed faint low-molecular-weight smears—typical of fragmented genomic DNA recovered from heat-processed meat matrices. Thermal processing (cooking/canning/autoclaving) is well known to fragment food DNA, in extreme cases leaving >90% of fragments short after severe treatment, which nevertheless remains amplifiable with short targets (Griffiths et al., 2023). This is in line with research by

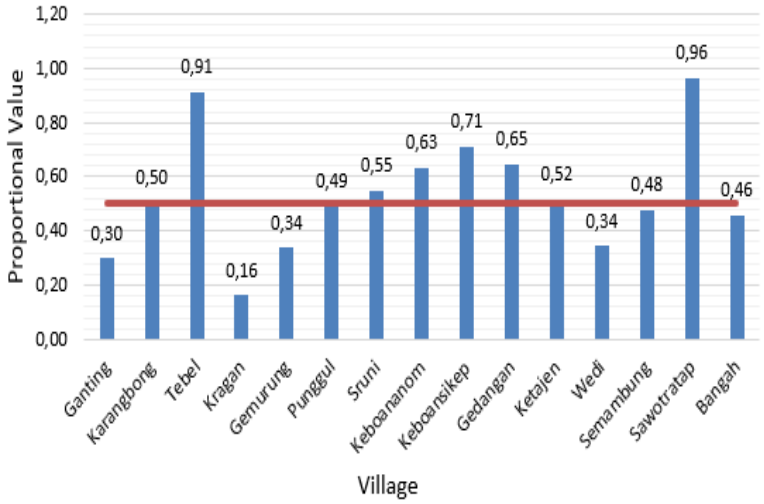

Figure 2. Selection of sampling strata by village in Gedangan District using stratified random sampling with a threshold value of 0.5

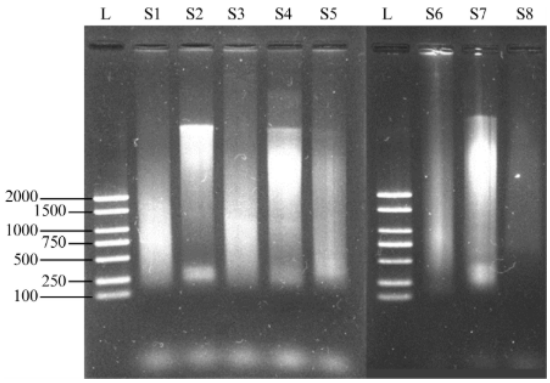

Figure 3. Visualized total DNA samples with 1% agarose electrophoresis; L= Ladder marker 2kbp from TINZYME DL2000+ DNA Marker (5 µL/T); S1–S8 = Total DNA from each sample.

Muhammed et al. (2015), which found that the treatment of the product heating process can degrade by 50-60%, 60-80%, and 70-90% at 80 °C, 100 °C, and 120 °C, respectively.

The visualization of total DNA was then quantified using a NanoDrop. The purity and concentration of DNAs will affect their quality. Table 6 shows that samples 1 to 8 have a purity level of 1.8-2.0, while the positive and negative controls showed purity levels of 2.13 and 2.03 at A260/A280. A pure DNA sample is indicated by the ratio of A260/A280 wavelength between 1.8-2.0 (Díaz et al., 2023). It was known that S1-S8 were pure DNA, while the positive and negative controls contained contaminants. These contaminants can be proteins, guanidine HCl, EDTA, carbohydrates, lipids, salt solutions, or phenols (Díaz et al., 2023).

The total DNA sample concentration ranged from 52.0 to 276.6 ng/µL, as shown in Table 6. From these results, it was found that S5 had the highest concentration value of 276.6 ng/µL, and the negative control had the lowest concentration of 52.0 ng/µL. The positive and negative control extracts were supplied by the laboratory and had been stored for ~1 month at -20 °C prior to our runs. DNA extracts are generally stable under such conditions. However, handling effects, notably freeze-thaw cycling and surface adsorption at low concentration, can reduce the measurable yield and/or increase fragmentation, leading to slightly lower apparent concentrations in stored controls than in freshly extracted samples (Finnis et al., 2023). Long-term storage at cold temperatures (-20°C) may cause DNA degradation of 0-20% within 30 days, 20-50% within 60 days, and 50-100% within 120 days (Muhammed et al., 2015).

Table 6. Total DNA concentration and purity

Sample	Purity λ 260 Å /280 Å	Concentration (ng/µL)
S1	1.82	179.00
S2	1.84	200.20
S3	1.82	229.70
S4	1.91	264.70
S5	1.90	276.60
S6	1.93	271.70
S7	1.83	193.40
S8	1.94	184.40
(+)	2.13	123.10
(-)	2.03	52.00

Jurnal Biodjati 10(2): 332–351, November 2025

JURNAL BIODJATI

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Polymerase Chain Reaction (PCR)

This research amplified a short mitochondrial cytochrome b fragment (~350–360 bp) using universal primers targeting conserved sites of mtDNA, widely used for food authenticity, even in processed matrices, because they still amplify fragmented DNA. Consequently, both porcine and beef DNA yield the same cytochrome b band, and species resolution is obtained at the RFLP step, or diagnostic restriction patterns on the amplicon, not from PCR alone (Azad et al., 2023; Farag et al., 2022). Based on the PCR amplicon visualization results in Figure 4, the positive and negative controls have DNA lengths between 300 and 400 bp. The DNA band length for cytb using the cytochrome b universal Forward primer CCA TCC ACC ATC TCA GCA TGA TGA AA and cytochrome b Reverse primer GCC CCT CAG AAT GAT ATT TGT CCT CA is known to be 359 bp (Fitriani, 2023). Therefore, the results for the positive (+) and negative (-) controls align with the expected band lengths. The blank control (BL) containing Nuclease Free Water (NFW) showed primer dimers. Primer dimers occur when primers self-bind without binding to the target template and appear as low-molecular-weight bands, typically <100–150 bp, often in the blank/no-template control (NFW), not at the target size (Khehra et al., 2025).

This primer dimer has the potential to interfere with the amplification process by causing non-specific amplification (Kim et al., 2023). Therefore, because the DNA primer sequence is similar to the primer dimer, this can increase the potential for false-positive results (Warmt et al., 2022). Samples 1 to 8 showed thin, blurred amplicons with smears across the bands. The bands from the samples were indicated to be around 300 bp to 400 bp in size. This indicates that the amplicons are close to the expected size of 359 bp for cytochrome b amplicons and are not false positives (Fitriani, 2023). The smear on the amplicon band may result from imperfectly paired bases. When subjected to electrophoresis, these imperfect paired bases cause a delay in electrophoretic migration within the gel, attributed to their secondary or tertiary structure (Zrimec et al., 2013).

PCR Optimization

Optimization of the polymerase chain reaction (PCR) protocol can be achieved by adjusting the annealing temperature (Zrimec et al., 2013). Adjusting the annealing temperature enhances primer binding specificity to the DNA template. An annealing temperature that is too low produces non-specific amplicons, while a temperature that is too high inhibits

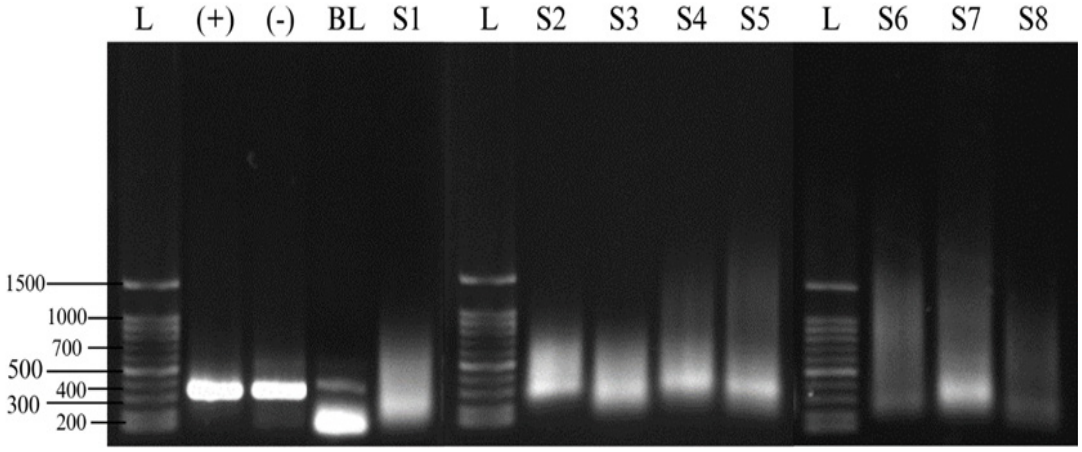

Figure 4. Visualized PCR amplicon with 1% agarose electrophoresis; L=Ladder marker 100 bp from TINZYME 100 bp DNA Ladder Marker DM10; (+)=positive control; (-)=negative control; BL blank control, S1–S8=amplicon of each sample

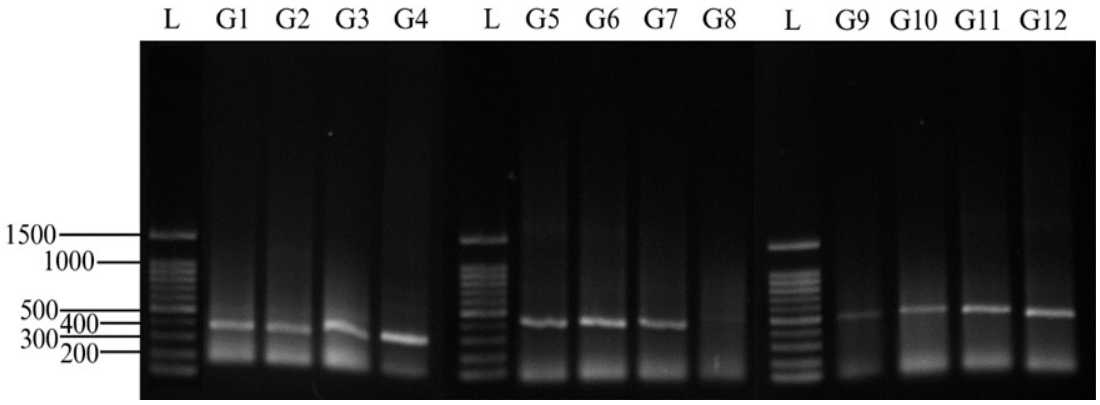

Figure 5. Visualized gradient PCR amplicon from optimization of annealing temperature using S4 total DNA sample with 1% agarose electrophoresis; L= Ladder marker 100 bp from TINZYME 100 bp DNA Ladder Marker; DM10. G1=60 °C; G2=60.3 °C, G3= 60.5 °C; G4=60.9 °C; G5=61.3 °C; G6=61,8 °C; G7= 62.2 °C; G8= 62.7 °C; G9= 63.1 °C; G10= 63.5 °C; G11= 63.8 °C; G12=64 °C.

primer binding and results in amplification failure (Yunita, 2023). The gradient PCR method enables systematic adjustment of the annealing temperature. The optimal melting temperature (Tm) for primers generally ranges from 50 °C to 65 °C (Masnaini et al., 2021). The annealing temperature of PCR program from Table 4 would be optimized using gradient PCR technique using the same template total DNA, which is S4 total DNA sample, across twelve temperatures with G1–G12=60.0–64.0 °C; G1=60.0, G2= 60.3, G3=60.5, G4=60.9, G5=61.3, G6=61.8, G7=62.2, G8=62.7, G9=63.1, G10=63.5, G11=63.8, G12=64.0 °C. Band clarity and intensity increased from G1 to G4, yielding a single sharp band at the expected size (~359 bp) (Figure 5) with minimal background at G4 (60.9 °C). Above G4, which are from G5 to G12, the band progressively faded and thinned, indicating excessive stringency that reduces primer–template binding. Based on (i) a single target-sized band, (ii) maximal intensity, (iii) absence of non-specific bands, and (iv) consistency across repeats (Khaira et al., 2023). Therefore, G4 (60.9 °C) was selected as the working annealing temperature.

Optimized PCR program validation

To validate the robustness around the optimum annealing temperature, we amplified S4 total DNA sample at G4 (60.9 °C), G6 (61.8 °C), G11 (63.8 °C), and G12 (64.0 °C) with temperature-matched blanks (BL4/BL6/BL11/BL12) and a positive control, which were showed in Figure 6. G4 (60.9 °C) consistently produced a single, strongest band at the expected size (~359 bp); G6 yielded a clear but weaker band, whereas G11 and G12 often gave weak/near-absent bands, a pattern consistent with excessive stringency at higher annealing temperatures reducing primer–template binding, while lower-than-optimal annealing favors some loss of efficiency or sporadic non-specific events (Latham et al., 2023). The blanks showed no band at the target size, where a faint smear/spot appeared close to the dye front (<150 bp), it is compatible with primer-dimers, a common low-molecular-weight by-product rather than true target amplification (Khehra et al., 2025).

Regarding the faint “shadow/shoulder” adjacent to the main band in some lanes (e.g., G4, G6, G11–G12): this is well-explained by Taq-mediated non-templated 3′ A-addition, which generates a mixture of +A and blunt isoforms that can resolve as closely spaced doublets on gels or electropherograms; this behavior has been explicitly demonstrated in recent analyses of Taq end-chemistry

Jurnal Biodjati 10(2): 332–351, November 2025

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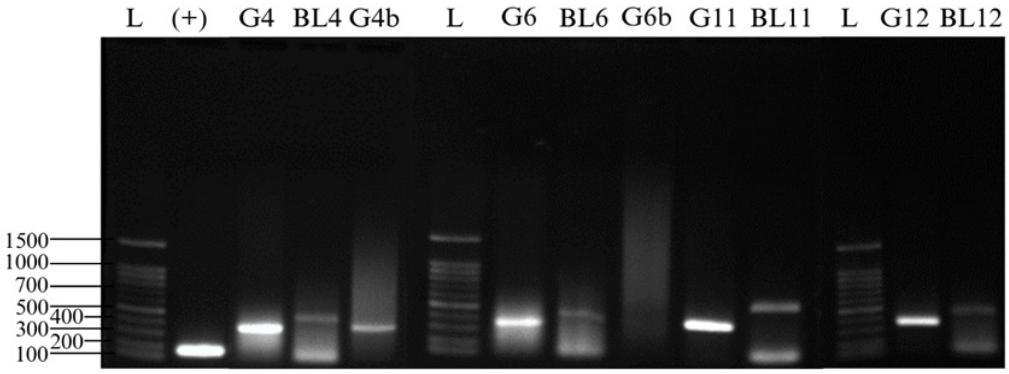

Figure 6. Visualized gradient PCR amplicon confirmation using S4 total DNA sample for optimalization of annealing temperature with 1% agarose electrophoresis; L= Ladder marker 100 bp from TINZYME 100 bp DNA Ladder Marker DM10; (+)=positive control; G4 and G4b=60.9 °C; BL4=blank in annealing temp. 60.9 °C; G6 and G6b= 61.8°C; BL6=blank in annealing temp. 61.8 °C; G12=64 °C; BL12= blank in annealing temp. of 64 °C.

(Zhou et al., 2023). A minor fraction of heteroduplexes can also form during post-PCR cooling (re-annealing of the +A/blunt isoforms), migrating slightly differently and appearing as a very low-intensity "ghost" near the principal band; heteroduplex formation in PCR products and its distinct migration are well documented and remain a recognized artefact in modern workflows (Murphy et al., 2024). Thus, the data support two conclusions: (1) the observed sample bands are true cytochrome b amplicons rather than false positives, and (2) G4 (60.9 °C) is the most reliable annealing temperature for subsequent PCR of field samples (S1–S8).

PCR optimum program

Based on the visualization of PCR amplicons at optimized temperatures shown in Figure 7, it was observed that all amplicon bands were within the range of 250 bp to 500 bp. This suggests that the CytB gene amplicon size, obtained from the positive control, negative control, and S1-S8 samples, was near the CytB band length of 359 bp (Fitriani, 2023). This band length falls under partial CytB, as the full CytB length is 1140 bp, which is typically used in DNA barcoding (Das et al., 2024). It is noted that partial CytB can also be used in phylogenetics as a mini-DNA barcoding method, with the advantage of being applicable to processed product samples, such as meat, which often have fragmented DNA (Elyasigorji et al., 2023).

Amplicon purification

The amplification product purification from agarose gel in this study was performed according to the protocol of the Mebep Bioscience Gel Extraction Kit, as described in the method. This step is essential for enabling successful downstream applications, such as restriction digestion, by producing high DNA yield and purity (Fatima et al., 2024). Then, based on the visualization results of the purified amplicons shown in Figure 8, it was observed that all purified amplicons from the samples and the positive and negative controls have the same length as the CytB band at 359 bp. Then, by analyzing the visualizations, the quality of the amplicon can be assessed: the presence of smears on the amplicon band indicates better DNA amplicon quality (Khamim & Qurrohman, 2024). Therefore, the purified DNA amplicons have better quality than the unpurified ones, as their smears were significantly reduced, resulting in clearer bands.

Restriction Fragment Length Polymorphism (RFLP)

The purified CytB amplicon was then digestedwith restriction enzyme BsaJI and

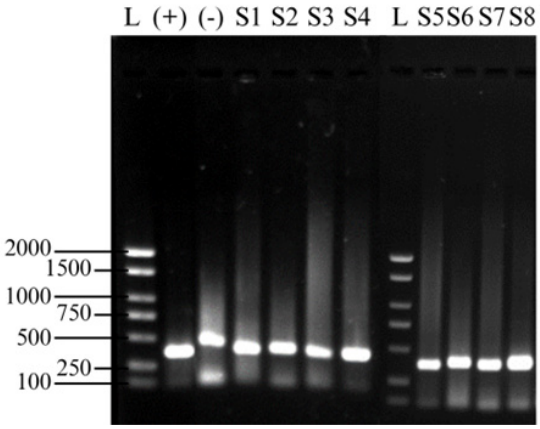

Figure 7. Visualized T-optimized PCR amplicon with 1% agarose electrophoresis; L=Ladder marker 2 kbp from TINZYME DL2000+ DNA Marker (5 µL/T); (+)=positive control; (-)=negative control; BL= blank control; S1–S8=amplicon of each sample.

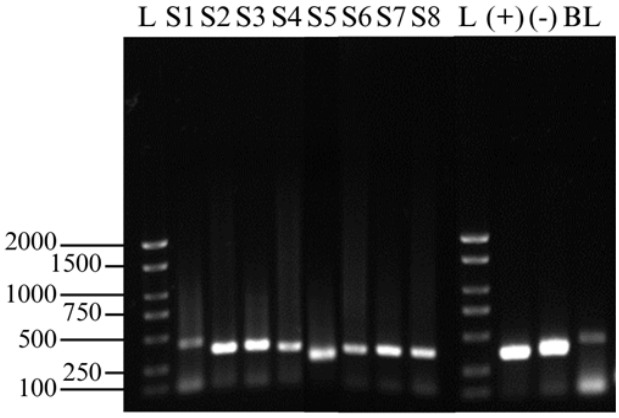

Figure 8. Visualized T-optimized PCR amplicon from purification with 1% agarose electrophoresis; L=Ladder marker 2 kbp from TINZYME DL2000+ DNA Marker (5 µL/T); (+)=positive control; (-)=negative control; BL=blank control; S1–S8=purified amplicon of each sample.

separated on 1% agarose, as shown in Figure 9. PCR-RFLP used for halal testing utilizes mitochondrial genes such as CytB, where the CytB gene contains a specific region unique to pork DNA, which can be cut by the restriction enzyme BsaJI (Hardianto et al., 2023).

The results of the amplicon digestion shown in Figure 9 indicated that all tested samples, S1 to S8, show a single amplicon band, suggesting that there was no digestion by the BsaJI restriction enzyme. This is consistent with the result of the negative control, which also shows only a single amplicon band. By contrast, the porcine positive control yielded the diagnostic multi-band pattern (two smaller fragments), confirming that the BsaJI restriction enzyme was active and can cleave the CytB amplicon from porcine DNA at the expected motif (Hays et al., 2022).

We deliberately loaded the positive control twice, once on each side of the sample lanes, to (i) demonstrate reproducibility of the cut pattern, (ii) verify BsaJI activity across the entire gel (left and right blocks), and (iii) provide an in-gel reference to compare migration near different sample groups. Moreover, apparent ifferences in band 'clearness'/intensity arise from routine gel factors rather than biology: (a) unequal loading (ng DNA per well) and slight differences in amplicon concentration, which we loaded half concentration of original G4 or G4b and G6 for G6b, (b) fragment size, the positive control's smaller diagnostic fragments naturally look more diffuse on 1%

Jurnal Biodjati 10(2): 332–351, November 2025

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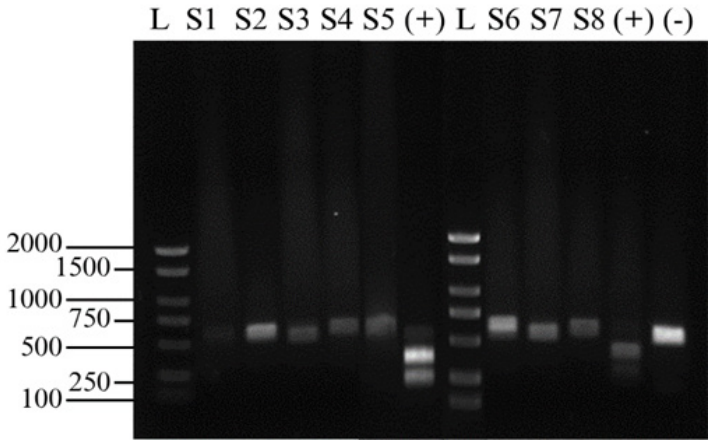

Figure 9. Visualized RFLP using BsaJI restriction enzyme's results with 1% agarose electrophoresis; L=Ladder marker 2 kbp from TINZYME DL2000+ DNA Marker (5 µL/T); (+)=positive control; (-)=negative control; BL=blank control; S1–S8=digested amplicon of each sample

agarose compared with a single band of 359 bp length, and (c) residual salts/detergent from PCR/digests that can broaden bands (Ramlan et al., 2024).

According to the user manual for the BsaJI restriction enzyme from TINZYME, the cleavage of the positive control amplicon occurs at the recognition sites 5'…C↓CNNGG…3' and 3'…GGNNC↑C…5', which is found in the CytB gene of pork. This is in line with in-silico RFLP using the Benchling Webserver, as shown in Figure 10—the cytochrome b amplicon from Bos taurus, acc. num. AY682377 remains uncleaved by BsaJI restriction enzyme with a band length of 360 bp, while from Sus scrofa, acc. num. DQ512916 was cleaved by the BsaJI restriction enzyme and forms two smaller bands with lengths of 228 and 131 bp. Thus, the results indicated that CytB from S1, S2, S3, S4, S5, S6, S7, and S8 does not contain BsaJI recognition sites, so all these samples were not contaminated with porcine DNA.

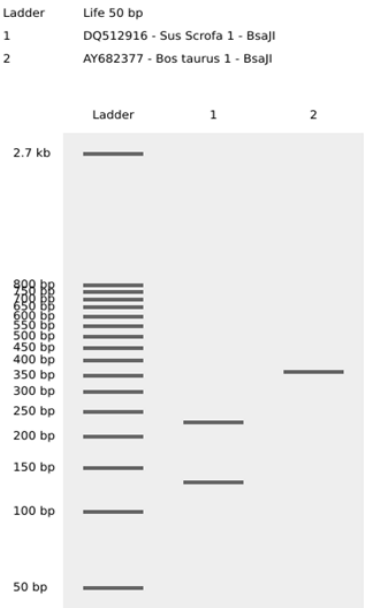

Figure 10. Restriction site of BsaJI restriction enzyme in the Cytochrome B amplicon of porcine (Sus scrofa, Acc. Num. DQ512916, 359 bp) and bovine (Bos taurus, Acc. Num. AY682377, 360 bp)

However, the amplicon results showed an increase in band size compared to the original CytB amplicon, which is 359 bp. The band sizes of the S1 to S8 samples and the negative control, which were initially in the range of 250 bp to 500 bp, shifted to 500 bp to 750 bp. This also occurred in the results of both positive control amplicons, where the fragment bands ranged from 250 bp to 500 bp. This change in size may occur due to the formation of secondary structures, such as G-quadruplexes, in the DNA bands, particularly during the RFLP incubation process (60 °C) at a temperature similar to the PCR annealing temperature (60.9 °C). Transcription, replication, or DNA repair processes can temporarily destabilize the DNA double helix, allowing the formation of G-quadruplex secondary structures in regions of the DNA strand rich in G nucleotides (Bryan & Baumann, 2011). The G-quadruplexes (G4) are G-rich nucleic acid sequences that fold into non-canonical secondary structures. G4s have two essential parameters: first, four strands (G-tracts) with G-residues form G-tetrads via Hoogsteen hydrogen bonding; second, these G-tetrads stack on each other and recruit monovalent cations (preferably Na^+ or K^+) (Grün & Schwalbe, 2021). G4s secondary structures that form tend to be more stable than DNA duplex structures because many G4s exhibit melting temperatures (Tm) above 60 °C and can exceed 70-80 °C depending on the sequences and ionic compositions. For example, the RET-promoter G4s show a Tm of ~86 °C in 50 mM K^+ but ~73 °C in 100 mM Na^+, indicating that G4 stability is strongly modulated by the Na^+/K^+ ratio (Zhang et al., 2024; Luo et al., 2024). Formation of G4s alters migration behavior in native gel electrophoresis: compact intramolecular G4 structures often migrate faster than their unstructured counterparts, whereas intermolecular/aggregated G4s species appear as slowly migrating or ladder-like bands. Mobility-based assays on native gels—including agarose EMSA—are routinely used to probe G4 formation and topology (Park et al., 2024; Cozzaglio et al., 2022; Lv et al., 2022).

The PCR-RFLP test detected that none of the retail meatball samples purchased from Gedangan District contained porcine contamination. To maintain this status and mitigate future risk, we recommend strengthening halal assurance systems across the supply chain, including but not limited to GMP, documented raw-material segregation and traceability, supplier verification, and scheduled molecular authenticity testing for high-risk products, rather than generic awareness campaigns.

CONCLUSION

The results of this study indicate that PCR-RFLP-treated samples from Gedangan Regency could not be cleaved by the BsaJI restriction enzyme, whereas the positive control was successfully cut. This finding suggests that all samples are free from porcine contamination. To maintain this status and mitigate future risk, we recommend strengthening halal assurance systems across the supply chain, including but not limited to GMP, documented raw-material segregation and traceability, supplier verification, and scheduled molecular authenticity testing for high-risk products, rather than generic awareness campaigns. This study is limited by a single-district, single-period sampling frame and a modest sample size; a single-locus, end-point PCR–RFLP design that is qualitative and depends on the presence of a BsaJI restriction site; and potential matrix effects of processed meat as well as secondary-structure–dependent mobility shifts on native gels that may influence band interpretation. Future work should broaden geographic and temporal coverage, adopt multi-marker and quantitative assays (e.g., qPCR/ddPCR or DNA barcoding), and include inter-laboratory validation to enhance robustness.

AUTHOR CONTRIBUTION

G.F.P.S. collected and analyzed the data and wrote the manuscript; **R.W.A.** collected the data; **L.N.F.** collected the data; **L.L.** reviewed and revised the manuscript and supervised all the processes.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript. The research was conducted and reported with full adherence to ethical standards and without any financial or personal conflicts that could have influenced the results or interpretation.

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