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# Successful Primer Picking and Pooling for the Design of Multiplex PCR Primers Specific to Pork, Beef, Chicken, and Rat DNA

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**ABSTRACT**

**DNA markers and Multiplex-PCR have emerged as methods for species detection in processed meat products. The primary objective of this study is to design multiplex primer sequences for pork, rat, beef, and chicken, generating distinguishable amplicons through agarose gel electrophoresis for halal detection in processed meat products. Primer design involved utilizing mitochondrial genomic data and the NCBI-Primer BLAST site to obtain specific pork and beef primer sequences. *In silico* simulations, including single and multiplex-PCR, were conducted using Primer Pooler. *In vitro* validation encompassed Single-PCR and Multiplex-PCR annealing temperature optimization, using samples of chicken, beef, pork, and rat as well as processed meat products like meatballs, sausages, and nuggets. *In vitro* validation demonstrated that the halal marker gene's multiplex primer efficiently amplified the target sequence, specifically at the optimal annealing temperature of 58°C. Amplicons from beef (1,217 bp), pork (860 bp), rat (622 bp), and chicken (272 bp) primers could be distinguished on a 1.5% agarose gel. The study's results can aid in cost-effective and rapid halal testing and authentication of processed meat products, offering advantages over PCR with a single primer.**

## 1. Introduction

The consumption of meat and processed meat products continues to increase worldwide (Ursachi *et al.* 2021), and Indonesia is no exception. This increase is because meat and processed meat products provide a source of high-quality protein needed for body activities (Hossain *et al.* 2020), the appearance of products, and the increasingly varied flavors of processed meat products (Sembor and Kowel 2012). In the consumption of processed meat products, some aspects are always considered by consumers, namely the halalness of processed meat products (Firdausi *et al.* 2020) because the majority of Indonesian people are Muslim, so processed meat products circulating in Indonesia must be labeled halal (Rachmawati *et al.* 2018). The Indonesian government has regulated the halalness of processed meat products (Mahbubi *et al.* 2019). However, there are still many producers who counterfeit processed meat products by mixing or changing the type of meat with non-halal meat, such as pork and rat, intending to reduce production costs and improve taste (Rahmania *et al.* 2015; Indriati and Yuniarsih 2019). This problem is certainly troubling to the public because the processed meat products consumed are difficult to distinguish with the naked eye (Riris *et al.* 2019). Therefore, species detection methods in processed meat products continue to be developed through DNA amplification techniques through Multiplex-PCR and genetic markers such as mitochondrial DNA (Alikord *et al.* 2017).

Mitochondrial DNA (mtDNA) is one of the DNA markers commonly used for species identification due to its high copy number and low recombination rate (Hossain *et al.* 2020). Multiplex-PCR is a more efficient amplification technique than conventional PCR (Kitpipit *et al.* 2014) because multiplex-PCR can amplify multiple target cycles (2-5 samples)

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simultaneously in one tube (Kim *et al.* 2016). Alikord *et al.* (2017) have successfully used m-PCR and DNA markers to detect horse, donkey, and pork contamination in halal-labeled processed meat products in Turkey. Indriati and Yuniarsih (2019) also used multiplex-PCR (m-PCR) from gene *Cyt b* to detect pork contamination in processed beef products in Indonesia. However, cases of adulteration of processed meat products in Indonesia are not only caused by pork contamination but also by rat meat contamination. Therefore, the use of multiplex-PCR and DNA markers for detecting rat and pork contamination in processed meat needs to be done.

The results of research by Dzikrina *et al.* (2022) have successfully used m-PCR and multiplex primers that produce amplicons with the size of chicken primer 272 bp, beef primer 522 bp, rat primer 622 bp, and pork primer 588 bp. However, the visualization of m-PCR products by electrophoresis on a 1.5-2.0% agarose gel showed that the amplicon from the pig, Beef, and rat primers could not be clearly distinguished. This is thought to be due to the difference in size of the primary amplicons of pigs, cattle, and rats <200 bp. Therefore, this study aims to redesign the DNA sequence of pork and beef primers with a difference in amplicons size ≥200 bp with chicken and rat primers to distinguish the resulting amplicons through agarose gel electrophoresis results. Then, multiplex primer combinations of halal marker genes will be used to detect the presence of rats, beef, pork, and chicken meat in multiplex on 1.5% agarose gel.

## 2. Materials and Methods

### 2.1. Primary Design

Beef and pork primers were designed using the mitochondrial genome sequences of cattle (*Bos taurus*) and pigs (*Sus scrofa*) from the NCBI Genebank site (https://www.ncbi.nlm.nih.gov/) with the "complete genome" category. In contrast, rat and chicken primers used the sequences from Dzikrina *et al.* 2022. After the Beef and pork primers were successfully redesigned, the sequence homology test was carried out with BLAST on the database contained on the NCBI website. Next, the Pick primer menu (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was selected by setting the amplicon length parameters and melting temperature (Tm) ranging from 57-63°C. Each primer sequence was analyzed based on primer standards, as shown in Table 1 (Shen *et al.* 2010; Ozturk and Can 2017). Homology tests were performed on standardized primers to ensure the primers were from the pig or cattle species used (Zangenberg *et al.* 1999).

### 2.2. *In-silico* Multiplex-PCR Simulation

Specific primer pairs were then compiled in fasta format (.txt) for use *in-silico* PCR simulations using PrimerPooler software (http://ssb22.user.srcf.net/pooler/). The simulation began with Single-PCR using pig and Beef primer pairs, which began by determining the presence of primer interactions through dG and threshold values with ≥-7 kcal/mol (Brown *et al.* 2017). Furthermore, the amplification process *in silico* was carried out using genomic data of four species combined with EmEditor software (http://www.emeditor.com). The amplification process is successful if the entire primer sequence is found in the genome and no overlaps occur. The cattle and pig primer sequences, successfully amplified through Single-PCR, were then used in Multiplex-PCR simulations with chicken and rat primers with the same procedure as Single-PCR. The best-simulatedd primer sequences were then ordered to Macrogen Korea. Primer requirements referring to Ozturk and Can 2017 and Shen *et al.* 2010 can be seen in Table 1.

### 2.3. DNA Extraction

DNA sample extraction was performed on meat samples and processed meat products using the Wizard Genomic DNA Purification Kit (Promega) with protocols for isolating and extracting DNA from animal cells or tissues. (Promega 2019). The isolated DNA was visualized through electrophoresis with 1% agarose gel in 1X TBE solution for 30 minutes

Table 1. Standard multiplex primers

| Parameter | Standard |
|---|---|
| Size | 18-27 bp |
| % GC content | 40-60% |
| Temperature melting (Tm) | 59-61°C (Difference ≤0.5°C) |
| Self-complementary | ≤ 8.00 |
| Self 3'complementary | ≤ 3.00 |
| GC Clamp | ≤ 3 |
| Repeats (Repetition of 2 bases) | ≤ 2 |
| Runs (repetition of bases) | ≤ 4 |
| Primer attachment | Species-specific/target-specific |

at a voltage of 50 Volts and visualized on a UV Transilluminator (Dzikrina *et al.* 2022).

### 2.4. Multiplex-PCR Optimization and Amplification

Amplification was performed through Single-PCR and Multiplex-PCR using a GoTaq® Green Master Mix 2X PCR amplification kit (Promega 2021). Single-PCR was performed as a positive control with a total reaction volume of 10 µL consisting of 5 µL GoTaq® Green Master Mix, one µL forward primer ten µM, one µL reverse primer ten µM, one µL DNA sample and two µL Nuclease free water. In comparison, the total Multiplex-PCR reaction volume was 25 µL consisting of 12.5 µL, eight µL forward and reverse primer mix, four µL DNA samples, and 0.5 Nuclease-free water. The negative control used dd$H_2O$. The PCR program was carried out based on the method of Qin *et al.* (2019) with modified annealing temperature optimization (Ta). The amplification process begins with pre-denaturation at 95°C for 5 minutes, then 35 cycles starting from the denaturation stage at 95°C for 30 seconds. The annealing stage is carried out at different temperatures, namely 55, 58, 60, 63, and 65°C for 30 seconds. Furthermore, the extension stage takes place at 72°C for 30 seconds at the initial stage and 5 minutes at the final stage, then holding at 4°C. The annealing temperature (Ta) of the best multiplex-PCR optimization results will be used to amplify processed meat products. Visualization of PCR results through electrophoresis with 1.5-2.0% agarose gel in buffer solution (1× TBE) using five µL of PCR products. Electrophoresis was carried out for 30 minutes at a constant voltage of 100 volts, and then the gel was visualized with a UV Transilluminator.

## 3. Results

### 3.1. Primer Design

Beef and pork mitochondrial genome data used in primer design from the NCBI website (www.ncbi.nlm.nih.gov) were selected with the complete sequence category for homology testing. BLAST results of pig mitochondrial complete genome (*Sus scrofa*, MK251046.1) obtained the top 100 complete mitochondrial DNA sequences of *Sus scrofa* and *Bos taurus* with homology values (percent identity) close to 100%. Percent identity of pig genome data contained in the NCBI site database between 98.74%-99.95% and genome length between 16689-16841 base pairs and for cattle (*Bos taurus*, GU947021.1) 99.89%-99.92% with mitochondrial genome length 16338-16341 pb.

Based on the results of the pig primer design on the NCBI-Primer BLAST site (Table 2), 13 of the 23 pairs of pig primers (860-950 bp) and 18 pairs of Beef primers (1,064-1,187 bp) were obtained, which had primer size, melting temperature, amplicons length, sequence, GC base content, self-complementary values, repeats, runs, and GC Clamp which were following the multiplex primer standards contained in Table 1.

### 3.2. *In Silico* Multiplex-PCR Simulation Results with Pooler

Based on the results of *in silico* simulations with Pooler, the ΔG threshold values of pork and beef primers in Table 3 show that all beef primer candidates have a ΔG threshold value of 0 kcal/mol. In contrast, the 1st, second, and third porcine primers have ΔG threshold values of -0.346 kcal/mol, 2.00 kcal/mol, and -0.042 kcal/mol, respectively.

Table 2. *In silico* analysis of pork and beef single PCR primer candidates

| Primer | Pick primer | | | | | | |
|---|---|---|---|---|---|---|---|
| | Contain GC (%) | Tm (°C) | Self-Compl | Self 3' Compl | Size of primer (bp) | Target gene | Amplicon (bp) |
| F_Pork1 | 60 | 59.97 | 5.00 | 0.00 | 20 | *ND5* | 860 |
| R_Pork1 | 55 | 59.96 | 2.00 | 2.00 | | | |
| F_Pork2 | 50 | 60.11 | 5.00 | 1.00 | 20 | *COX3* | 861 |
| R_Pork2 | 55 | 60.03 | 7.00 | 0.00 | | | |
| F_Pork3 | 55 | 60.04 | 4.00 | 2.00 | 20 | *ND2* | 862 |
| R_Pork3 | 55 | 59.96 | 4.00 | 2.00 | | | |
| F_Beef1 | 55 | 60.11 | 3.00 | 2.00 | 20 | *ATP8* | 1,095 |
| R_Beef1 | 55 | 60.08 | 6.00 | 0.00 | | | |
| F_Beef2 | 55 | 59.96 | 3.00 | 2.00 | 20 | *ND5* | 1,217 |
| R_Beef2 | 55 | 59.96 | 2.00 | 1.00 | | | |
| F_Rat* | 55 | 60.03 | 5.00 | 3.00 | 20 | | 622 |
| R_Rat* | 55 | 60.03 | 4.00 | 3.00 | | | |
| F_Chicken* | 55 | 60.04 | 4.00 | 1.00 | 20 | | 272 |
| R_Chicken* | 55 | 59.97 | 3.00 | 3.00 | | | |

*Dzikrina *et.al.* 2022

Table 3. *In silico* analysis of pork and beef single PCR primer candidates with pooler

| Primer | Pooler | | | |
|---|---|---|---|---|
| | ΔG (kcal/mol) | Threshold (kcal/mol) | Amplicon single-PCR | Overlap |
| F_Pork1<br>R_Pork1 | 0 | -0.346 | 870 | No |
| F_Pork2<br>R_Pork2 | 0 | -2.00 | - | Yes |
| F_Pork3<br>R_Pork3 | -2 | -0.042 | 871 | No |
| F_Beef1<br>R_Beef1 | 0 | 0 | - | Yes |
| F_Beef2<br>R_Beef2 | 0 | 0 | 1202 | No |
| F_Rat*<br>R_Rat* | 0 | -0.377 | 638 | No |
| F_Chicken*<br>R_Chicken* | 0 | 0 | 278 | No |

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Single-PCR simulation results using pig and Beef primers in Table 3. show that only the first and third pig primer sequences were successfully amplified without overlap and produced 870 bp amplicons. The second Beef primer produced 1,202 bp amplicons.

Table 4 shows that multiplex primers' dG and threshold values show lower values than single primer dG values and produce dimer forms. The dG threshold value generated from the first primer set is -1.49 kcal/mol, while the dG threshold value generated is -2.09 in the second primer set.

Multiplex-PCR simulation results *in silico* with the first and second primer sets showed that eight primer sequences from different species were found in the mitochondrial genome and successfully attached to the target sequence in the genome without overlap, resulting in amplicons with the most extended size of 1,217 bp.

### 3.3. Multiplex-PCR Optimization Results

Figure 1 illustrates Single-PCR amplification at various annealing temperatures, showing successful primer attachment with single DNA bands. At 55°C and 58°C, cattle and chicken bands were thinner than pig and rat bands, while at 60°C, 63°C, and 65°C, bands were thicker and almost equal in thickness. Multiplex-PCR amplification produced bands only at 58°C, 60°C, and 63°C, with no bands at 55°C and 65°C. Successful amplification at 58°C, 60°C, and 63°C displayed distinguishable multiplex bands from four species, although band thickness decreased with higher annealing temperatures.

Table 4. *In silico* analysis of PCR multiplex primers with pooler

| No | ΔG (kcal/mol) | Threshold (kcal/mol) | Amplicon m-PCR | Overlap |
|---|---|---|---|---|
| Primer set 1 (Chicken, rat, Beef2, pork1) | -1 | -1.49 | 1217 | No |
| Primer set 2 (Chicken, rat, Beef2, pork3) | -2 | -2.09 | 1217 | No |

### 3.4. Amplification of Pork, Beef, Rat, and Chicken Marker Genes in Processed Meat Products

An optimal annealing temperature of 58°C was used to amplify ten processed meat samples. All samples, labeled pork and beef-based, unexpectedly contained chicken DNA in the *COX1* region. The chicken band appeared thick and distinct in all samples, but thinner in the pork sausage sample. Despite product labels indicating non-chicken content, the amplification results revealed the presence of chicken DNA in wells 8, 9, 10, 12, and 13 (Figure 2).

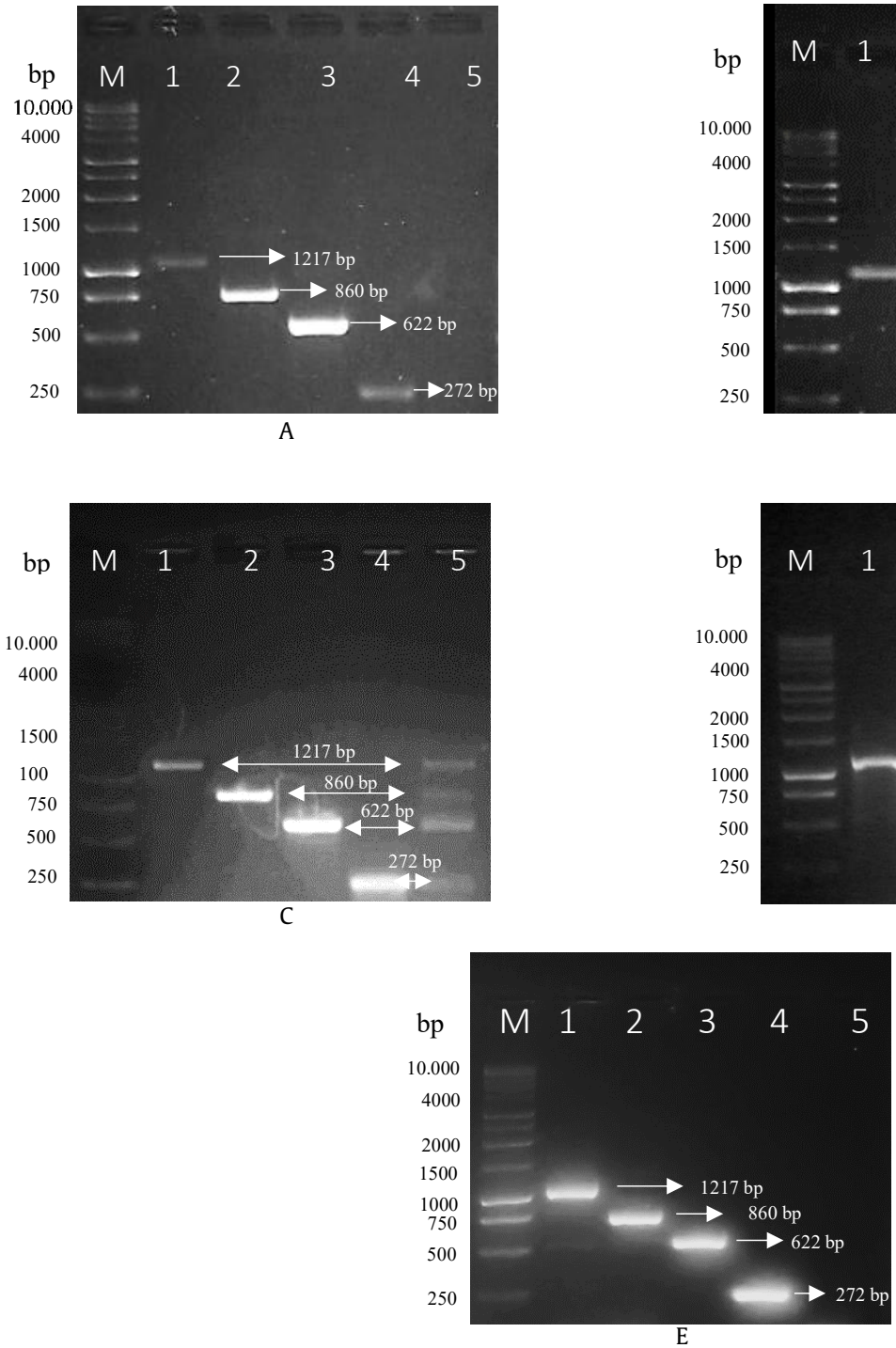


Figure 1. Multiplex-PCR Temperature Optimization Results (A) 55°C, (B) 58°C, (C) 60°C, (D) 63°C, (E) 65°C, M: ladder, 1: beef, 2: pork, 3: rat, 4: chicken, 5: multiplex

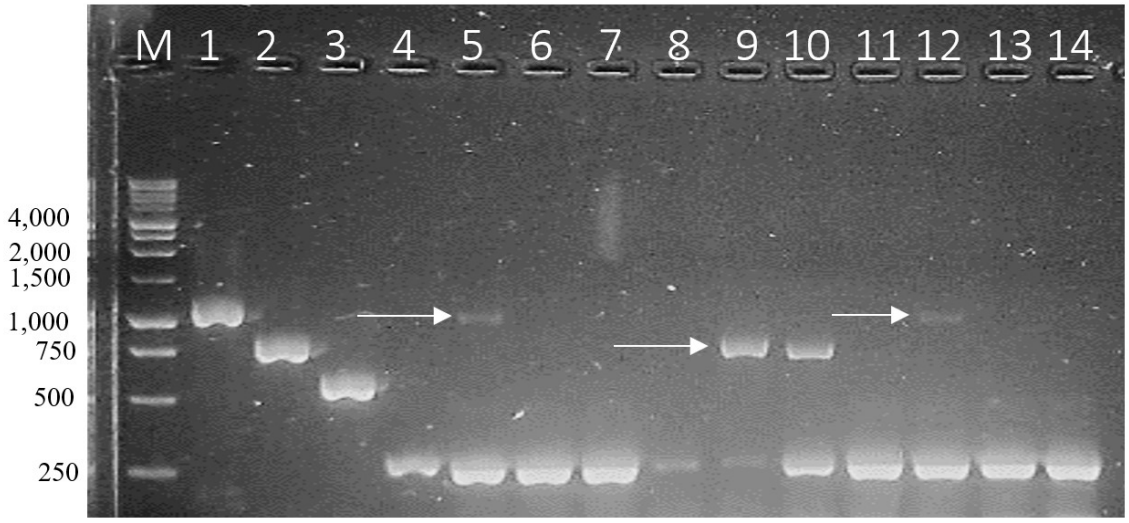


Figure 2. Multiplex-PCR results of processed meat products M: ladder, 1: beef, 2: pork, 3: pat, 4: chicken, 5-9: branded products (5: combination meatball, 6: combination sausage, 7: chicken nuggets, 8: pork meatballs, 9: pork sausage), 10-14: bulk products (10: pork sausage, 11: chicken meatballs, 12: beef meatballs, 13: beef sausage, 14: chicken nuggets)

## 4. Discussion

Blast nucleotide results showing nearly 100% homology suggest suitability for later pork and beef primer design. Tindi *et al.* (2017) defines homologous sequences by similar Max and Total scores, near 100% Query Coverage, E-values close to 0, and Percent Ident near 100%. This genome data was used for primer design.

All candidate primers underwent a crucial homology test to exclude those outside pork and cattle species. Multiplex-PCR employs four pairs of primers from different species; if pork and beef primer sequences are also found in chicken and mouse genomes, it could lead to errors. According to Mahony and Chernesky (1995), Multiplex-PCR primers must specifically bind to the template DNA of the desired species to avoid errors during co-amplification. Selected primer candidates will undergo *in silico* PCR simulation using Primer 3 and Pooler.

The initial simulation assesses primer candidates based on the dG threshold value, representing the Gibbs energy needed for secondary structure breakdown (Pratiwi *et al.* 2019). The delta G values range from 0 to -2 kcal/mol, indicating a moderate likelihood of strong primary interactions. Eling *et al.* (2014) suggest a suitable delta G value for primer structure release is more than -6 kcal/mol, as excessively negative values can impede the annealing process. Primer Pooler's standard protocol considers a delta G value and threshold of -7 kcal/mol tolerable (Brown *et al.* 2017). Consequently, pork and beef primers are suitable for the Single-PCR simulation stage.

From Table 3, two candidate multiplex primer sets were identified for chicken, rat, and beef primers: multiplex primer set one with pork1 primer and multiplex primer set 2 with pork2 primer. *In silico* Multiplex-PCR analysis assesses the ΔG and threshold values of these sets. The analysis reveals that a low ΔG value in Single-PCR results corresponds to a lower Multiplex-PCR ΔG value. For example, in the first primer set, the ΔG value of the pork1 primer is lower than that of the pork2 primer, making the ΔG value of primer set 2 more negative than primer set 1. Despite this, both primer sets fall within the acceptable tolerance limit of ≥-7 kcal/mol (Brown *et al.* 2017), permitting their use in the amplification stage.

The longest amplicons, measuring 1,217 bp, are likely beef amplicons, aligning with Primer Pooler standards where successful amplification occurs in the absence of dimers or when dimer values are near zero. All primers matched their respective genomes, were non-overlapping, and yielded amplicons of defined sizes (Brown *et al.* 2017). These findings suggest the primer set is suitable for *in vitro* validation. The pork_1 primer from multiplex primer set 1 will be used for *in vitro* validation due

to its higher and closer to zero ΔG value, which is expected to minimize dimer formation.

The key outcome of the Single-PCR process is producing a single band, confirming that the designed primer specifically binds to the target DNA and generates amplicons of the expected size. This aligns with Nejad *et al.* (2014) research, where single-PCR results demonstrated primer specificity through a single band correlating with the designed amplicon size.

At 55°C and 65°C, no bands appeared in the multiplex amplification results. This could be due to imperfect primer attachment at too low or too high temperatures under the same reaction conditions. Additionally, the Single-PCR results at 55°C exhibited the thinnest bands, particularly for beef and chicken primers, indicating unsuitable temperatures for primer attachment in both Single-PCR and Multiplex-PCR, resulting in no amplification products.

Low annealing temperatures, as noted by Green and Sambrook (2019), may result in incomplete primer attachment to the target sequence due to interactions between primers. Conversely, high annealing temperatures can impede primer attachment in Multiplex-PCR, reducing product yield (Zangenberg *et al.* 1999). Suboptimal primer concentration, a crucial factor highlighted by Henegariu *et al.* (1997), can also contribute to unsuccessful amplification in Multiplex-PCR.

Clear and distinct amplicon bands on a 1.5% agarose gel confirm the specific recognition of target regions by designed primers, including the *COX1* gene in chicken and rat samples and the *ND5* gene sequences in pork and beef samples. Successful amplification at three temperatures indicates suitable conditions for primer attachment, with the most precise and thickest band observed at a 58°C attachment temperature, demonstrating optimal conditions for Multiplex-PCR. The thinner band at higher annealing temperatures suggests difficulty in primer attachment, in line with Green and Sambrook's (2019) recommendation to maintain an optimal annealing temperature by reducing the melting temperature. Chen *et al.* (2021) also identified 58°C as the optimal annealing temperature in quadruplex-PCR research using four pairs of primers.

The Multiplex-PCR results at three attachment temperatures align with the study's objectives, demonstrating successful primer attachment to target sequences in chicken, pig, rat, and beef DNA samples. The optimal temperature is found to be 58°C, resulting in thicker and more precise bands, and will be applied for rapid detection in processed meat products. The presence of chicken bands in all samples is attributed to the common use of chicken eggs in processed meat products, as noted by Alamsyah *et al.* (2019). Consequently, all samples tested positive for chicken DNA.

In well number 8 (pork-based meatball) and well number 13 (beef sausage), neither beef nor pork species were detected, suggesting a potential falsification of product labels. This finding aligns with Nejad *et al.* (2014) research, where chicken DNA was detected in sausage samples labeled as beef. In contrast, well number 5 (chicken and beef meatballs) and well number 12 (beef sausage) showed the detection of beef and chicken DNA, similar to the detection of pork and chicken DNA in pork sausage samples (wells 9 and 10). These results demonstrate the multiplex primers' ability to specifically identify multiple DNA species, revealing potential mislabeling in processed products.

Cytochrome c oxidase 1 (*COX1*) and *ND5* are mitochondrial genes used for species identification due to their distinctive, conserved regions and variations between species, making them ideal for differentiating species (Izadpanah *et al.* 2018; Li *et al.* 2020). Mitochondrial DNA (mtDNA) is advantageous in processed meat product detection due to its abundance, circular double-stranded structure, and resilience to processing Nejad *et al.* (2014). Multiplex-PCR is key in this detection, allowing simultaneous amplification of four target sequences in one reaction, even from small or complex samples. This study's main achievement is the successful design of primers that identify specific DNA sequences from chicken, pig, rat, and beef in a single reaction, distinguishable through agarose gel electrophoresis. These findings hold promise for rapid, efficient halal testing of processed products.

The multiplex primer set (beef, pork, rat, and chicken) successfully enabled Multiplex-PCR, generating distinguishable amplicons (1,217 bp beef, 860 bp pork, 622 bp rat, 272 bp chicken) detectable through agarose gel electrophoresis. This designed gene multiplex primer set offers a fast detection method for identifying pork, beef, rat, and chicken in processed meat products, aiding species identification in the community's processed meat.

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