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Multiplex PCR for simultaneous detection of DNA contamination from non-halal species in beef products

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Abstract

A large number of mixtures of non-halal ingredients such as pork and rat meat in processed foods has worried the public, especially adherents of the Islamic religion. There is a need for a method to detect the presence of non-halal contaminants in several foods found in the community. This study aims to obtain a valid method by proposing multiplex PCR to detect DNA in processed meat foods. The sample of this research was processed beef (meatballs) obtained from five parts of the region from Surabaya, Indonesia. Multiplex PCR results on target species (pigs, mice, cattle) showed thick and clear DNA bands. This indicates that the amplification runs optimally. The designed primer can be used to test samples of processed meat foods on the market. The results showed that there was no adulteration with rat meat or pork in all samples in all areas in Surabaya. The results of electrophoresis showed that there was only one DNA band measuring 495 bp which was the result of the amplification of bovine DNA. The multiplex PCR developed in this study proved to be effective for detecting non-halal contaminants in meat-processed food products to be used as a reference for other tests.

1. Introduction

No composition list, mislabelling, or mixing of non-halal ingredients is a serious problem and often harms consumers (Cai *et al*., 2017; Khatun *et al*., 2021). The ones who are most affected by this problem are Muslims who have an obligation to consume halal food and drinks (Hassan *et al*., 2018). The number of Muslims in the world is expected to increase by 73%. Reach around 30% of the total world population by 2050 (Ali *et al*., 2017). This population growth encourages an increase in total halal food consumption in the world (Lee *et al*., 2021). The turnover of the world's halal food industry has exceeded USD 661 billion (Ali, Hashim, Mustafa *et al*., 2012) and has grown rapidly even for non-Muslim consumers due to consumer protection and perceived benefits and significantly reduces the risk of developing zoonotic diseases (Abdullah and Azam, 2020).

Beef is one of the most widely consumed sources of animal protein (Montalvo-Puente *et al*., 2018). Products that are very susceptible to counterfeiting are beef and its products because the characteristics of beef are similar to red meat in other types of animals such as pork. The grinding process of several types of processed beef products such as meatballs also opens up more gaps to mix with other red meat. The colour, taste, and shape are similar to beef, with a much cheaper price are the strong reasons for using pork as a mixture in processed beef (Regenstein *et al*., 2003). Rat meat is also often used to mix beef products because the price is cheap and can even be obtained for free. The more advanced processing and packaging technology makes it difficult or even impossible to identify food components, especially processed foods based on their physical attributes alone (Ali, Kashif, Uddin *et al*., 2012).

Using nucleic acid as an approach for authenticating meat products is the best method because it is more stable and resistant to the cooking process than lipid or protein biomarkers. PCR with species-specific primers that are designed appropriately with optimized formulations is the most appropriate method for detecting and identifying species in a product, this method can also reduce costs because it does not require the digestion of restriction or sequencing of PCR products (Karabasanavar *et al*., 2014). However, compared to conventional species-specific PCR (simplex) systems, testing with multiplex PCR with species-specific primers is very promising (Ali *et al*., 2015). Multiplex PCR contains several primers sets with a single PCR reagent mixture to produce amplicons of varying sizes specific to different DNA of species targets

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simultaneously (Hayden *et al.*, 2008). This research aimed to find the right primer combination for multiplex PCR of cattle, pigs and mice which will later be used in the detection of beef products sold in the market.

2. Materials and methods

2.1 DNA extraction

Approximately 20 mg of the sample was ground using a mortar and pestle. Then, DNA extraction is done in accordance with the GeneJET Genomic DNA Purification Kit protocols. The quantity and quality of extracted DNA were measured using the Thermo Scientific™ Multiskan GO UV/Vis microplate spectrophotometer. DNA content was measured at an absorbance of 260 nm and purity was determined based on the absorbance ratio of 260 and 280 nm. A ratio of 1.8 to 2 indicates good DNA purity.

2.2 Primers design

Species-specific primers are designed to target mitochondrial genes because mtDNA is well protected by the mitochondrial membrane, is inherited maternally and exists in multiple copies per cell (Dong *et al.*, 2019). Between the mitochondrial genes, cytochrome-b (CYTB) and NADH dehydrogenase subunit 5 (ND5) have the right target length, the appropriate intra-species grade area, inter-species polymorphism, and a sequence database. The sequences of the two genes from the three species were downloaded to GenBank and then aligned. Primers that have been designed are also screened for their specificity to eliminate cross-species binding with other animal and plant species using the online Basic Local Alignment Tool (BLAST) with the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3 Singleplex PCR

Primers were tested with the target DNA of each species separately. The PCR composition used consists of a mixture according to the Thermo Scientific DreamTaq Green PCR Master Mix protocol with the total volume per reaction was 25 µL with the composition of each reaction is 12.5 µL master mix, 1.25 µL of each forward and reverse primers, 1 µL DNA template, and 9 µL ddH_2O. The PCR reaction was carried out in a pre-denaturation stage of 94ºC for 3 min, followed by 35 cycles of denaturation, annealing, and extension with 94ºC, 50ºC, and 72ºC respectively 30 s, and ended with a final extension at 72ºC for 5 min.

2.4 Duplex and multiplex PCR

Duplex PCR is done as a first step to test the specificity of the primer by placing two target species in one reaction. Three PCR reactions were performed, namely for primers and samples from pigs and mice, primers and samples from pigs and cows, and primers and samples from rats and cows. Multiplex PCR is done by putting all three target species in one reaction so that simultaneous amplification can occur. The cocktail and cycle used are the same as for the singleplex PCR. All results of the PCR reaction were visualized using 1% agarose gel electrophoresis and captured using the Syngene Gel Documentation System.

2.5 Sample collection

To determine the optimized multiplex PCR capability, direct testing was carried out on samples of processed meat on the market. Meatballs are the most widely consumed processed meat in the Indonesian market, especially Surabaya. Meatball samples from street food meatball and meatball shops were taken from 5 regions in Surabaya, such as South, East, West, North, and Central Surabaya. From each region, three samples were taken randomly.

3. Results

3.1 Specificity of singleplex PCR

Singleplex PCR was conducted to test the specificity of the primers and also as an optimization step of the annealing temperature of each primer. The annealing temperature used in this PCR is 50ºC. This temperature was chosen because it produces the most optimal DNA band.

In Figure 1, it can be seen that there are three DNA strands of different sizes. The size of each band is in accordance with the size of the amplicon produced during the primary design stage, a pig with 235 bp, rat 238 bp and cow 495 bp. This shows that the primers used are suitable and can be continued for the next analysis.

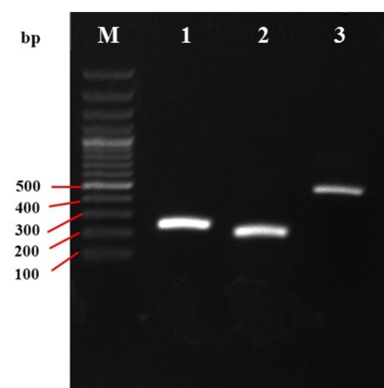

Figure 1. The results of 1% agarose electrophoresis were from singleplex PCR. M: DNA ladder 100 bp; 1: pig; 2: rat; 3: cow.

3.2 Specificity of duplex and multiplex PCR

Duplex PCR is conducted to test the primary specificity before multiplex PCR is carried out. The

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results in Figure 2 showed that the primer is specific and also there is the absence of mispriming in the amplification process. This is indicated by the presence of two DNA bands that matches the targeted product and no other non-specific product is produced.

The multiplex PCR results are shown in Figure 2 indicated by line 4 which indicates the presence of 3 DNA bands. This indicates that multiplex PCR amplification targeting cow, mouse, and pig species has been successfully carried out. The absence of mispriming and non-target DNA bands due to non-specific amplification, and the thick and clear band results also indicate that the amplification is running optimally.

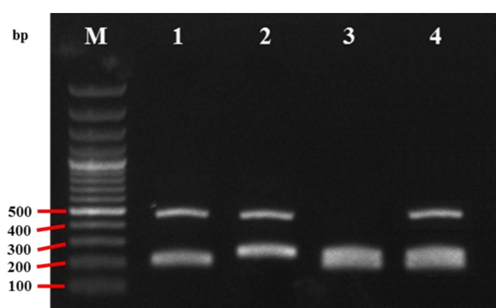

Figure 2. The results of 1% agarose electrophoresis were from duplex and multiplex PCR. M: DNA ladder 100 bp marker; 1: cow and pig; 2: cow and rat; 3: rats and pig, 4: cow, rat, and pig.

3.3 Testing samples by multiplex PCR

Primers that have been tested for specificity with single, duplex and multiplex PCR are then used to test processed beef products in Surabaya, Indonesia. Figure 3 -7 is the results of multiplex PCR from processed meat in the north, east, south, west, and central Surabaya.

The results showed that there was no adulteration with rat meat or pork in all samples in all areas in Surabaya, which appears to be only one DNA band in size of 495 bp as the result of beef DNA amplification. Besides showing the presence of bovine species in the product, the presence of this cow DNA band also indicates that the amplification process has occurred.

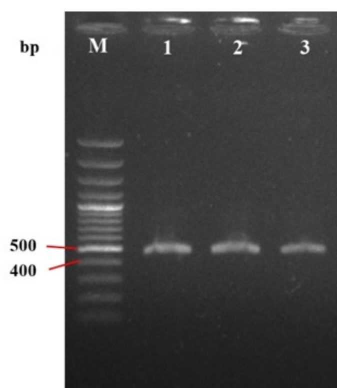

Figure 3. The results of multiplex PCR electrophoresis on processed meat in North Surabaya. M: DNA ladder 100 bp marker; 1-3: samples of processed meat.

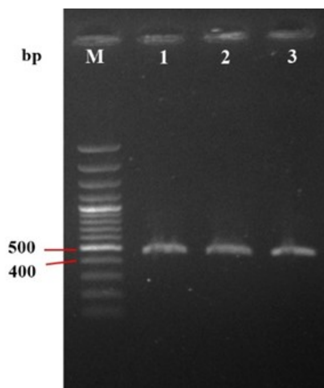

Figure 4. The results of multiplex PCR electrophoresis on processed meat in East Surabaya. M: DNA ladder 100 bp marker; 1-3: samples of processed meat.

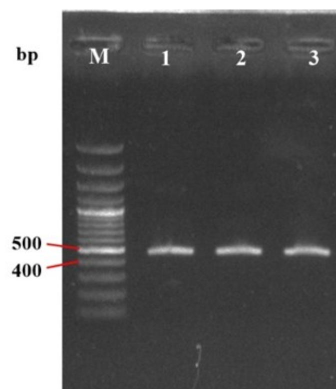

Figure 5. The results of multiplex PCR electrophoresis on processed meat in South Surabaya. M: DNA ladder 100 bp marker; 1-3: samples of processed meat.

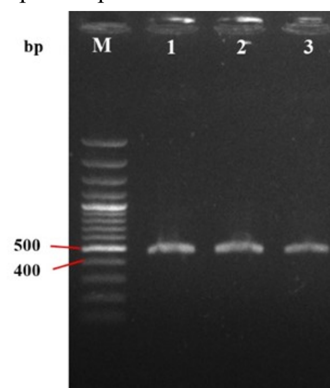

Figure 6. The results of multiplex PCR electrophoresis on processed meat in West Surabaya. M: DNA ladder 100 bp marker; 1-3: samples of processed meat.

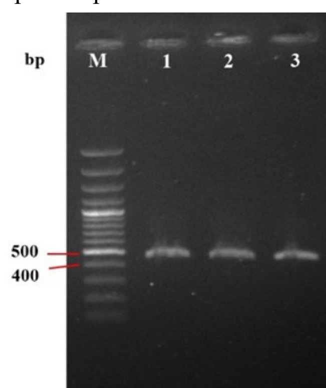

Figure 7. The results of multiplex PCR electrophoresis on processed meat in Central Surabaya. M: DNA ladder 100 bp marker; 1-3: samples of processed meat.

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4. Discussion

Halal is an important thing for a Muslim to choose food. The halal food industry covers the entire process from upstream to downstream. But sometimes there are halal food scams along the food supply chain to get more profit (Ruslan *et al*., 2018). Counterfeiting of products involving the substitution of meat ingredients is often done by producers. This counterfeiting resulted in certain religious and moral complaints involving the substitution of beef with non-halal meat. Identification of pig contamination in food products is very necessary to provide protection to Muslim consumers (Soon *et al*., 2017).

The PCR technique is very appropriate for testing the composition of meat and processed meat because it only requires very small amounts of DNA (Che Man *et al*., 2007). In addition, the difficulty of detecting the composition of meat in cooked samples does not prevent PCR from accurately detecting it. DNA traces still exist in the sample even though it has been degraded and can still be used as a template for the amplification process

Primer design in multiplex PCR is very crucial because, in addition to the effectiveness of simultaneous reactions, the same annealing temperature but species-specific, and also different amplicon sizes are the keys to the success of multiplex PCR. In addition to the annealing temperature, optimization also needs to be done in the volume and composition of the reaction as well as the PCR cycle (Ali *et al*., 2015).

The products of duplex and multiplex PCR on pig and mouse DNA bands can be separated well in 1% agarose gel but the results cannot be seen clearly in the gel documentation, this is because agarose cannot separate DNA whose length difference is less than 40-50 bp (Bottero and Dalmasso, 2011). This can be improved in the future by keeping the difference in product sizes so that when visualization can be seen clearly. However, it is still necessary to pay attention to the relationship between annealing temperature and primer specificity with one.

The majority of the Indonesian population are adherents of the Islamic religion (Widyaningrum and Yu, 2018). Every Muslim has an obligation to eat halal food, meaning that it is free from contamination of non-halal materials such as pork and rats (Septiani, 2019). One of the efforts of the Indonesian government to provide services to the community is to certify the food in circulation. Foods that pass the halal certification are allowed to include the halal logo on the packaging (Faridah, 2019). Until 2019, products that have been certified halal are still around 10%. This indicates that there are still many foods circulating in the community but the halal status is not yet known.

The authenticity of food ingredients is very important to prevent fraud and food safety (Ruslan *et al*., 2018). This study shows that multiplex PCR can be used as an appropriate method for the detection of non-halal contaminants in processed meat products. All of the samples tested in this study did not contain non-halal ingredients so they were safe for consumption, especially by the Muslim community. There is a need for synergy and cooperation from various parties to maintain the integrity of the halal products distributed in the community. In addition, proactive measures are also needed for food counterfeiting.

5. Conclusion

Protection of health, religious needs, budgets, and consumer choices must be a commitment to all food producers and governments. The simultaneous detection of three species for beef products is intimately a choice to save up to three times the cost of testing. The multiplex PCR developed in this study successfully amplified the three species. Meat processed samples in the market with processing in high temperatures of the cooking process and a high risk of DNA degradation were also successfully tested using the multiplex PCR method developed in this study.

Conflict of interest

The authors declare no conflict of interest.

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