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Comparison of ELISA and PCR Assays for Detection of Pork Adulteration in Halal-Labelled Beef Products

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HIGHLIGHTS

- Qualitative Enzyme-Linked Immunosorbent Assay could detect pork contain in heated beef product.
- Pork specific primers with a band length of 531 bp may identify pork combinations.
- Polymerase Chain Reaction method should be evaluated for intensive heat-treated samples.

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Acronyms and abbreviations
ELISA=Enzyme-Linked Immunosorbent Assay
OD=Optical Density
PCR=Polymerase Chain Reaction

ABSTRACT

Background: Food adulteration with pork in processed beef products is one of the most serious issues in a food sector in a Muslim-majority country since it is related to religious food ethics regarding the halal products. The goal of this research is to test the suitability of ingredients in beef floss and its Halal by knowing the presence of pork DNA and protein in those products.

Methods: Meat products were prepared from two famous marketplaces in Indonesia labeled contain beef meat. In this study, a qualitative Enzyme-Linked Immunosorbent Assay (ELISA) test was compared to a conventional Polymerase Chain Reaction (PCR) assay to determine pork adulteration in beef floss.

Results: The results of the ELISA test showed that two products labeling Halal and containing beef ingredients were positive for pork. Those two samples continued testing using conventional PCR assay. The result of the conventional PCR assay was negative for those two samples.

Conclusion: It may be helpful to utilize both traditional PCR and ELISA for species detection due to the possibly inhibiting compounds contained in some processed meat products. The results of this research suggest that ELISA is better than conventional PCR method for product samples that have received an intensive heating process.

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Introduction

The marketplace is a platform that facilitates online product transactions between customers and suppliers. Some processed food products have no label information such as trademarks, manufacturing dates, and expirations, the composition of the materials used even Halal logos are not listed on the product packaging so that the possibility of substitute of other materials can occure. The most important types of food cheating are the products components of the food as known as adulteration product (Al-Taghlubee et al., 2019). Food ingredients, packaging, and brand misrepresentation, sometimes known as mislabeling, are examples of various food cheating

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(Banti, 2020).

Substitute ingredients or adulteration products is a problem that is frequently found in processed meat products such as beef floss. Pork is a common alternative ingredient in beef floss products since it is very inexpensive, has a similar colour and shape to beef, and so lowers production costs while increasing flavour. The act of adulterating products with pork mixes will be extremely harmful to the community in Muslim countries such as Indonesia, which has a predominantly Muslim population of around 207,176,162 people (Hasan, 2019).

In Indonesian society, the concept of Halal has been adopted in people's lives. According to Islamic sharia, Halal refers to something Halal and permissible for humans to eat or consume (Habibie et al., 2019). Halal is an Islamic concept that incorporates cleanliness, safety, purity, virtue, manufacturing, production, procedure, honesty, truth, and food service, as well as other financial and social activities (Hussain et al., 2016). Halal food, according to Islamic law, is food that is free of pork, khamar, and other banned substances. Pigs and other forbidden objects should also not be engaged, according to Islamic teachings on processing, storage, processing, and food equipment. The quality changes to become haram when Halal products are combined with haram products.

Pork mixture in processed beef floss is difficult to distinguish directly but can be identified with the lab analysis. One of the efforts that can be made to identify the presence of pork contamination as a guarantee of food security is to develop methods of health analysis and the reliability of a product. Test methods that can be used to detect the presence of pork contamination at this time include Polymerase Chain Reaction (PCR) (Al-Kahtani et al., 2017; Pestana et al., 2010; Soares et al., 2013) DNA hybridization (Ballin et al., 2009), Enzyme-Linked Immunosorbent Assay (ELISA) (Asensio et al., 2008; Kuswandi et al., 2017) and Liquid Chromatography-Mass Spectrometry (LC-MS) (Kleinnijenhuis et al., 2018). Protein-based ELISA test techniques and DNA-based PCR test techniques are methods often used in the detection of pork. The genetic indicator mithocondrial DNA and cytochrome B can be used to identify pig contamination in meat and processed meat by duplex PCR, according to a prior study by Ni'mah et al. (2016).

The risk of adulteration pork as beef in circulating beef products needs to be monitored to ensure safe, healthy, whole Halal, and competitive animal products. This study used protein tests such as ELISA and DNA tests such as PCR test to identify and analyze pork content contamination in processed beef floss products in order to determine which methods can produce more sensitive and accurate results.

Materials and methods

Study design

Processed beef products were prepared from two Indonesian marketplaces, namely 3 samples from T store and 3 samples from S store where the samples packaged with labels listed such as ingredient composition, expiration date, and logo that reads 100% original beef. Six samples of beef floss were coded namely T1, T2, and T3 for three samples purchased from T and S1, S2, and S3 for 3 samples purchased from S. Each beef floss sample from each store was prepared for 25 g for testing with ELISA method and 25 mg for testing with PCR method using analytical scales. Positive controls were pig blood samples, while negative controls were aquadest, cow, and sheep blood samples. Blood samples were collected from farms in and around Yogyakarta.

ELISA

The sandwich ELISA was used according the standard procedures for testing the Porcine Detection Kits for processed meat (Biokits Neogen Corp., USA). Extraction of the sample was done by homogenizing 25 g of the sample with 100 ml of physiological sodium chloride (NaCl), then heated by boiling at a temperature of 95-100 ºC for 15 min and left at room temperature for 15 min. Then, the filtered and centrifuge sample was then taken 100 µl lower layers for further test using ELISA. Every ELISA test was accompanied by positive and negative controls. Each sample tested by ELISA was repeated twice. The reading of the results was done with ELISA Reader (Biochrome EZ Read, USA) at a wavelength of 450 nm by looking at the Optical Density (OD) value. Determination of positive or negative test results was done by comparing OD values and cut-off values. The cut-off value was obtained by the formula:

Cut-off=The average amount of negative controlx2.5 (multiplier factor)

If the OD value was higher than the cut-off value, the sample tests was considered positive. If the OD value was lesser than the cut-off value, the sample was considered negative (Biokits Neogen Corp., USA).

DNA extraction

According to the manufacturer's instruction, DNA was extracted from beef floss using Quick-DNA™ Universal Kit (Zymo Research, USA). On the other hand, DNA was isolated from blood following by FavorPrep™ Tissue Genomic DNA Extraction Mini Kit (Favorgen biotech corp, USA). DNA templates were stored at -20 °C until the next analysis.

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PCR

The PCR amplification was conducted in the total volume of 25 µl containing 15 µl ddH2O, 5 µl master mix (5X PCR Master Dye Mix, ExcelTaq, SMOBIO, Taiwan), 1 µl primer, and 4 µl DNA template. The positive controls of pig blood samples and the negative control of aquadest, cow, sheep blood samples, and beef product samples were used. Two specific primers for pig designed by Montiel-Sosa et al. (2000) were used in this study; the forward primer: 5′-AACCCTATGTACGTCGTGCAT-3' and the reverse primer: 5'-ACCATTGACTGAATAGCACCT-3'. The PCR reaction was carried out using a thermal cycler (SeletCycler II Thermal Cycler, Select BioProducts, USA) as follows: initial denaturation at 94 ºC for 2 min, followed by 30 cycles of denaturation at 94 ºC for 30 s, annealing at 58 ºC for 30 s, extension at 72 ºC for 40 s; final extension was applied at 72 ºC for 5 min.

Electrophoresis and vvisualization of PCR pproduct

PCR amplification were assessed using 1.5% agarose gel electrophoresis (GeneDireX, Taiwan) in Tris-borate-EDTA (TBE) buffer (Omnipure, Merck, USA) containing 1 X FluoroVue DNA staining (FluoroVue, Smobio, Taiwan). PCR products and a 100-bp DNA ladder (Smobio, Taiwan) were placed in each well. Using underwater electrophoresis equipment, the gel was electrophoresed for 23 min at 135 V (Mupid-exU, Japan). A Dual Light Emitting Diode (LED) Blue Transilluminator (BIO-HELIX, Taiwan) was then used to illuminate the gel. After that, the PCR result bands were captured using a camera and compared to a DNA ladder.

Results

Presence of pork was detected in 2 of 6 samples of beef products. Two positive samples, namely SA1 and SA2, had absorbance values of 0.574 and 0.519. That result was greater than the cut off value of 0.452, while the four negative samples of SA3, TA1, TA2, and TA3 had absorbance values 0.287, 0.174, 0.225, and 0.198. The cut off value was calculated by multiplying the average negative control value, 0.181 by a factor of 2.5.

The specific primer used under the selected conditions amplified the pig gene with an expected band of 531 bp. Following DNA isolation from beef floss samples without checking the quality of DNA due to limited tools. The results of PCR visualization using a double LED blue transilluminator (Figure 1) showed that there were no positive pork products in the beef floss samples (S1, S2, S3, T1, T2, and T3). Result of meat species identification using ELISA and PCR are compared in Table 1.

Table 1: Result of meat species identification using Enzyme-Linked Immunosorbent Assay (ELISA) and Polymerase Chain Reaction (PCR)

Sample	Meat ingredients on label	Halal- label	PCR	ELISA
Control+	-	-	+	-
Control-	-	-	-	-
Control-	-	-	-	-
S1	Beef	-	-	+
S2	Beef	-	-	+
S3	Beef	-	-	-
T1	Beef	-	-	-
T2	Beef	-	-	-
T3	Beef	-	-	-

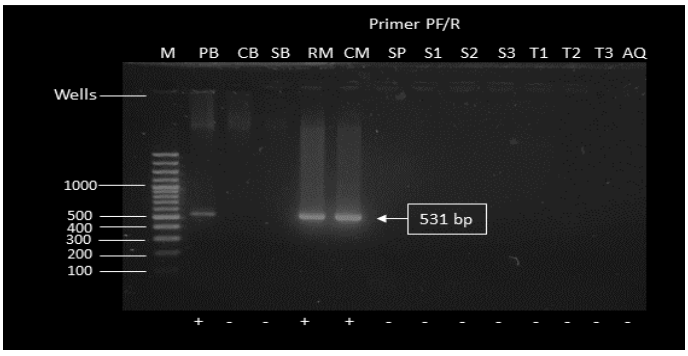


Figure 1: Result of Polymerase Chain Reaction (PCR) conventional method visualization. M: 100 bp ladder; PB: Pig blood;CB: Cattle blood; SB: Sheep blood; RM: Raw pig meat; CM: Cooked pig meat; SP: Sredded pig; S1 to S3: beef floss sample from S; T1 to T3: beef floss sample from T, and AQ : aquades.+: Positif -: negatif.

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Discussion

Sandwich ELISA and indirect ELISA are the most often used ELISA methods in the study of food components (Asensio et al., 2008). Sandwich ELISA was carried out qualitatively which was easy and fast in its application and had good sensitivity. Sandwich and indirect ELISA technology have a thermal stable-soluble protein that is good for detection of raw and processed pork at low concentrations (Asensio et al., 2008; Kim et al., 2016). In this study, it was shown that 2 out of 6 (33.33%) of the beef floss samples purchased from two marketplaces in Indonesia were contaminated with pork. Yörük (2021) observed the presence of pork in 19 out of 30 (63.3%) samples of various processed products that had been heated such as salami, sausage, and ham. A similar study was also conducted to examine the sensitivity of the indirect ELISA method using HRP conjugated anti-pig igG polyclonal antibody with artificial samples of pork which were adulterated into beef in the concentration range of 1, 5, 10, 25, 50, 100 (%w/w) and the results allow detection of pork contamination at a concentration level of 0.1% (Mandli et al., 2018). According to research carried out in Kosovo, 3% of the chicken-based food samples had a moderate level of pork adulteration and 5% had a low level of pork adulteration, whereas 4% of the beef based food samples had a moderate level of pork adulteration and 28% had a low level of pork adulteration (Gecaj et al., 2021).

The results of this study using the conventional PCR technique showed that there was no pork content in the beef floss samples tested. This might occur due to technical problems such as failing to verify the quality of DNA extraction due to the limits of the equipment employed, which could impact the outcomes of the DNA amplification reaction and prevent the visualization findings from being created appropriately. Additionally, the process of processing shredded beef by heating at high temperatures and for a long time can cause DNA loss. In comparison to other PCR procedures, the traditional PCR used in this work has low stability. The initial stage in molecular biology research is DNA isolation. The quality of the DNA template must be assessed in order to gauge the quality of the DNA that was successfully extracted since it can impact the process of DNA amplification reactions (Wardana and Mushlih, 2021). Similar research found that a band of mtDNA could be successfully amplified from meat cooked by various methods, including boiling, roasting, and pressure cooking, except for pan-frying, and that an indistinct band could be obtained after normal cooking but no band could be seen after excessive pan-frying (Arslan et al., 2006). As a result, more testing utilizing more stable PCR technologies, such as real-time PCR or duplex PCR, is required. Ni'mah et al. (2016) from Indonesia used the duplex PCR approach to detect pork in both fresh and cooked beef products. Similar research revealed that 4 out of 17 (23.53%) samples of meat items offered in supermarkets included pig when it was evaluated using direct lysis multiplex PCR (Zhao et al., 2021). According to research carried out in South Africa using the real-time PCR technology, 4 of the 21 (19.05%) samples of canned food products with the "no pork" claim on their packaging were found as containing pork (Tantuan and Viljoen, 2021).

The identity of meat species is a crucial problem from a health and regulatory standpoint, and food safety and quality are crucial topics. ELISA test techniques with protein-based methods and DNA-based PCR testing techniques have been used to detect unwanted food content. Both techniques are capable of detection adulteration in many types of raw meat and some processed foods (Perestam et al., 2017). Similar research showed that real time PCR using pork-specific primers in conjunction with commercial ELISA kits provides an appropriate and cost-effective testing and monitoring method in retail marketplaces in Kosovo using pork meat in chicken and beef based commercial products as sampels (Gecaj et al., 2021). Spending through the marketplace at this time began to increase; unfortunately, there are still many stores that sell processed meat products such as a product called beef floss which on the packaging does not include Halal labels so that questions arise in the community about the page of the product. According to Hasan (2019), the Halal logo on a product is acknowledged as a symbol of cleanliness, safety, and high quality; hence it should be on a processed food product's label. Therefore, it is necessary to standardize and certify Halal products using laboratory analysis using both methods above to achieve proper quality assurance of Halal food products and maintain the safety of consumers.

The ability to identify pigs using both methods (Table 1) showed that the ELISA method was more stable than the conventional PCR method to detect pork contamination in processed meat products such as beef abon after heating treatment at high temperatures for a long time such as beef floss. The ELISA method is good to use as a routine test because it can be used for large samples, relatively fast time, and quite sophisticated equipment but at an affordable price (Asensio et al., 2008). On heating treatment and the addition of sodium nitrate, NaCl, phosphates, citrates, and ascorbates, ELISA has good stability against an antigenic epitope (Zvereva et al., 2015). Confirmation with laboratory tests for more accurate species identification, both protein-based and DNA analysis are necessary to avoid false-negative or false-positive results. The conventional PCR approach is a DNA or genetics-

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based analysis that is significantly more heat-stable than a protein-based test (ELISA), thus it cannot detect pork in processed meat with high heating temperatures, such as beef floss. Test analysis based on genetic detection such as PCR need requires skill, time, and sophisticated laboratory equipment so that the costs incurred are quite high. Perestam et al. (2017) according to the findings of the study that real-time PCR was shown to be more difficult to perform and to take more time than ELISA .

In terms of Halal awareness, it is well known that most Muslim consumers in Indonesia already have a basic concept of what constitutes Halal food; yet, they are unaware that not all processed foods marketed are Halal. Because the majority of Indonesians are Muslims, many consumers expected that all items offered would be Halal. However, not all processed food companies are Muslim. Furthermore, in the past, identification of the processing and raw materials utilized in the food sector was simple. As previously stated, the origins of this enhanced awareness originated in 1989, when the swine oil issue was brought to public attention.

The case shocked Indonesian society up from its lengthy slumber. In addition, the Ajinomoto case in 2001 taught Indonesian society that obtaining Halal food items is not as simple as they assumed, because food is produced through a high-tech and complex food engineering process. It's easy to tell the difference between Halal and non-Halal food when it's processed with a simple procedure and contains evident raw materials. However, the difficulties in delivering Halal items to fulfill market demand has increased since the growth of food science, which has also had the effect of shifting people's preferences toward improved flavor and quality.

Conclusion

From the analysis of this study, the Halal label and the content of the ingredients do not guarantee the authenticity of the contents of the product. Even though one test method states a negative result, it is necessary to be careful because another test method states a positive result. When testing items containing additional components, such as beef floss, it may be helpful to utilize both traditional PCR and ELISA for species detection due to the possibly inhibiting compounds contained in some processed meat products. The results of this research also suggest that ELISA is more dependable, quicker, and simpler to use than PCR tests. However, the PCR test is less costly to carry out when compared to ELISA. It seems that ELISA approach is better than the conventional PCR method for product samples that have received an intensive heating process.

Author contributions

P.Ap., R.U., C.M.A., F.A., and P.As. were responsible for the experiment design and data analysis and also did all experiments and drafted the manuscript; P.Ap., R.U., and F.A. participated in the evaluation of each experiment; P.Ap., R.U., and F.A. revised the paper and provided technical support and the final edition of the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

All the authors declared that this is no conflict of interest in the study.

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