Molecular Detection of Pork Contamination in Beef Sold in Traditional Markets, Yogyakarta

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ABSTRACT

Beef is one of animal protein sources needed by the body to meet nutritional requirements. Consequently, fresh beef (whole or milled) is one of the highest-demanded comestibles on the market. Some meat kiosks in Yogyakarta traditional markets are also on service of milling whole meat. However, there are reports stating that some kiosks are neglecting aspects concerning halal. One of those aspects is separating tools used to process beef and pork. Reports said that kiosk owners fail to meet that basic requirement causing Muslims to be at risk of consuming contaminated beef without their knowledge. Hence, this study aims to examine whether fresh and ground beef obtained from Pasar Pathuk and Pasar Kranggan, Kota Yogyakarta are free from pork contamination. Examination was carried out using Polymerase Chain Reaction (PCR) on 14 samples consisting 7 fresh beef and 7 ground beef obtained from both markets. DNA isolation from all samples were done using FavorGen® FavorPrepTM Tissue Genomic DNA Extraction Mini Kit. Isolated DNA was further examined by PCR analysis using P14 and MTCB primers. Results showed that P14 primers could amplify PRE-1 gene (481 bp) designed as pork molecular marker only on positive control (fresh pork) while MTCB primers could amplify cytochrome b gene (1141 bp) designed as mammal molecular marker on all samples involved in this research. Based on the results, we concluded that both fresh and ground beef sold in Pasar Pathuk and Pasar Kranggan, Yogyakarta are not contaminated by pork DNA.

1. Introduction

The vast majority of Indonesian population is Muslim. As a Muslim-majority country, Indonesian government is obliged to ensure that food circulating in the community are safe, of good quality, nutritious and halal-certified (Triasih *et al.,* 2016). One of halal certification criteria requires that food must be free from any pork elements as stated in the Quran (An-Nahl:115), *"He has only forbidden you to eat carrion, blood, swine, and what is slaughtered in the name of any other than Allah. But if someone is compelled by necessity—neither driven by desire nor exceeding immediate need—then surely Allah is All-Forgiving, Most Merciful."*. Meat is the source of animal protein necessary for the body to meet nutritional needs. Meat is often milled to be the main ingredient for many meat-processed food products (e.g. sausages, meatballs, and corned beef). Many people who do not have milling machine at home would go to meat kiosks in the markets that offer milling

service. Quoted by Redaksikibar (2013), there were several meat kiosks that offer milling service in Pasar Pathuk and Pasar Kranggan that violated the basic rule of providing halal comestible; separating tools used to mill pork and beef. This becomes a substantial problem because it is a transgression on someone's belief and an offense to Law Number (No). 8 of 1999 on Consumer Protection. Technological advances in conducting analysis on pork contamination have increased. One of those advances is the use of *Polymerase Chain Reaction* (PCR) to identify the presence of pork elements on meat-processed food. Fibriana *et al.* (2012) conducted a study using PCR to identify pork contamination on meatballs sold on the street of Salatiga, Central Java. One of thirteen samples of this study was found contaminated by pork meat. Similar study has been conducted in Surabaya using RT-PCR. In that study, there were 5 samples out of 30 tested positive for pork contamination on milled beef (Susilowati, 2019). However, there is limited literature on molecular identification of pork meat contamination in Yogyakarta hence this research was conducted. Fourteen samples were used in this study. Those samples were taken from Pasar Kranggan and Pasar Pathuk, Kota Yogyakarta.

2. Materials and Methods

This research was conducted in January – April 2021 in the Laboratory of Biochemistry and Biotechnology, Universitas Ahmad Dahlan Yogyakarta. All samples were collected from Pasar Kranggan and Pasar Pathuk, Kota Yogyakarta. Fresh pork, fresh beef, and ddH_2O were used as controls in this research.

2.1 Sample Preparation and DNA Extraction

Samples taken from traditional markets were transferred to laboratory using cooler box. From each sample, as much as 30 mg were milled and transferred into 1.5 ml microtube. 200 µl FATG1 buffer, proteinase K was added into the microtube. Samples were then vortexed. Samples were incubated in 60◦C for 1-3 hours. After first incubation, 200 µl of FATG2 buffer was added and samples were incubated for the second time for 10 minutes. After the second incubation, 200 µl of 96% ethanol was added and samples were vortexed. FATG mini columns were transferred into collection tube and centrifuged for 1 minute (18,000 rpm). Collection tube was discarded and replaced by new collection tube. 400 µl of W1 buffer was added into the column. Samples were then centrifuged full-speed for another 1 minute. Collection tube was discarded and was replaced by new collection tube. 750 µl wash buffer was added into the column and was centrifuged full-speed for 1 minute. Collection tube was discarded and replaced by new 1,5 ml microtube. 100 µl elution buffer was added into the column and the column was incubated in room temperature for 3 minutes. Samples were then centrifuged for 2 minutes. Pellet formed after centrifugation was stored in 4◦C.

2.2 Qualitative and Quantitative Analysis of DNA

Qualitative analysis was carried out using 1.5% agarose gel immersed in 0.5x TAE buffer solution. 3 µl of DNA sample, 2 µl loading dye, and 5 µl GelRed were used for every well in agarose gel. Electrophoresis tank was set to 100 volts and 15 minutes. Observation of agarose gel was carried out on UV transilluminator to detect DNA bands. Quantitative analysis was carried out using Nanodrop.

2.3 PCR Amplification

PCR was used to amplify target gene using two pairs of primers as shown in Table 1. P14 primers design was adapted from Fibriana et al. (2012) and MTCB design was adapted from Naidu et al. (2012). As for PCR condition is shown in Table 2. As much as 1.2 µl DNA sample, 12.5 µl *MasterMix*, 1 µl primer forward and 1 µl primer reversed were mixed into each of the PCR tube. 9.3 µl ddH_2O was added into the tubes. The tubes were then placed into PCR machine. PCR products were then stored in -20°C for further use.

Table 1. Primers Sequence

Primer	Sequence
P14F	5'-CCCCGTCTCCTTCCTCCGGTGGTTGATG-3'
P14R	5'-CTGCGACACATGATGCCTTTATGTCCCAGC-3'
MTCBF	5'-CCHCCATAAATAGGNGAAGG-3'
MTCBR	5'- WAGAAYTTCAGCTTTGGG-3'

Table 2. PCR Condition

Condition	Temperature	Duration	Cycle
Pre-denaturation	95°C	2 minutes	-
Denaturation	95°C	45 seconds	
Annealing	55°C	1 minute	
Extension	72°C	1 minute	30 cycles
Final extension	72°C	5 minutes	

3. Results and Discussion

This study aims to identify pork DNA in beef (whole and milled) obtained from Pasar Pathuk and Pasar Kranggan using *polymerase chain reaction* (PCR). This method has a high sensitivity to detect the smallest amount of DNA present in the samples of interest. In this study, two pair of primers were used; MTCB *(mitochondrial cytochrome b)* and P14.

DNA isolation was done using FavorGen commercial kit *FavorGen® FavorPrep™ Tissue Genomic DNA Extraction Mini Kit*. DNA isolation consists of 3 main principles: cell destruction (lysis), separation of DNA from solid materials such as proteins and cellulose (extraction) and DNA purification (Nurhayati and Darmawati, 2017). Isolated DNA on fresh pork, fresh beef, whole and ground beef were visualized using gel electrophoresis as a qualitative measure. Results of visualization are shown in Figure 1.

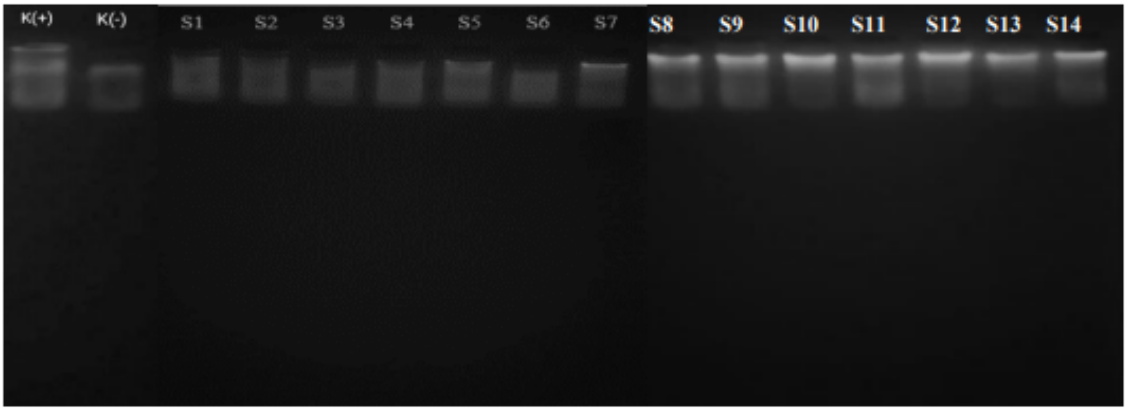

Figure 1. The result of fresh meat DNA isolation. K(+): Positive control (fresh pork); K(-): negative control (fresh beef); S1 - S7: fresh beef; S8 – S14: ground beef.

Based on Figure 1, it is shown that DNA quality of S8 – S14 samples were better compared to S1 – S7 samples. One factor that could explain this is the contaminants in the form of proteins or the remaining solution from the isolation kit (Kurniama et al. 2017 and Fatchiyah et al. 2011). Smears that appear on the bands are caused by at least two factors. First factor is the remnants of solution from the isolation kit, second factor is DNA degradation during isolation (Setiaputri et al. 2020 and Suparningtyas et al. 2018). Repeatedly washing DNA with ethanol could possibly clear the smear, therefore can be used as one method to purify DNA (Fatmawati et al. 2015). Samples were then further analyzed by Nanodrop as quantitative measure to obtain DNA concentration; results are shown in Table 3.

Table 3. Measurement of Quality and Concentration of DNA Isolates

Sample	**A260/A280 Ratio**	**Concentration of DNA (ng/µl)**
Positive Control	1,746	103,07
Negative Control	1,993	87,07
S1	2,018	162,68
S2	1,905	144,11
S3	1,741	73,74
S4	1,818	186,45

S5	1,987	75,37
S6	1,805	96,71
S7	1,976	100,63
S8	1,971	114,47
S9	1,993	209,16
S10	1,834	114,85
S11	1,945	157,06
S12	1,946	135,85
S13	1,832	45,65
S14	1,868	114,90
Average	**1,807**	**108,985**

The measurement of DNA concentration and purity is necessary to know the degree of contamination of the isolated DNA. DNA of good quality would have A_{260}/A_{280} ratio on the range of 1.8 to 2.0. A_{260}/A_{280} ratio below 1.8 indicates that DNA contains protein contaminant. Failure to break down (lysis) cell components could be the cause of protein contaminant (Pratama, 2015). A_{260}/A_{280} ratio above 2.0 indicates that DNA contains remnants of the RNA. Measurement of DNA concentration is also necessary for the next step of analysis, which is PCR. That is because there is a certain amount of sample concentration that need to be met to obtain high quality of amplicons (Mustafa et al., 2016 and Fatchiyah et al., 2011). Average A_{260}/A_{280} ratio of 1.807 shown in Table 1 indicates that purity of DNA isolated in this research was of good quality.

Considering the good result of DNA concentration, samples were further analyzed by *polymerase chain reaction* (PCR). Figure 2 shows PCR products using MTCB primers. Based on the results, all samples, control meat included, were all successfully amplified on ~1140bp.

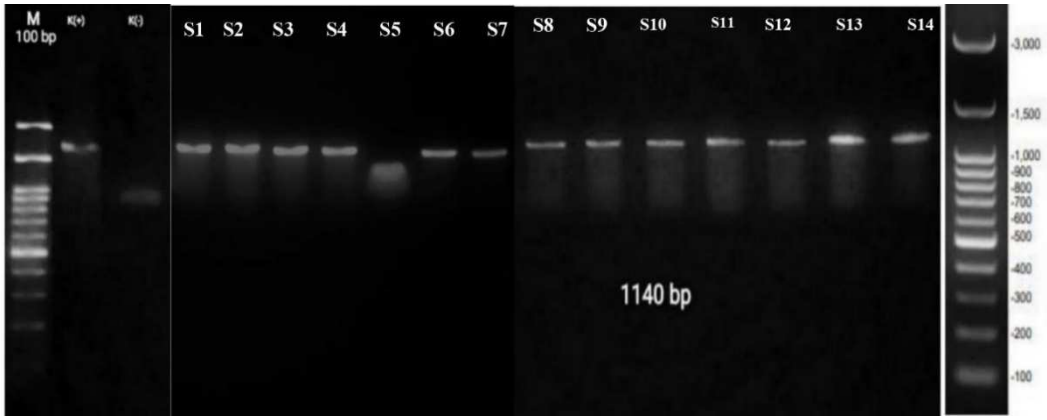

Figure 2. PCR amplification results using MTCB primers electrified in 1.2% agarose gel 100 volts; M: marker 100 bp; K(+): positive control (fresh beef); K(-): negative control (ddH2O); S1 - S7: fresh meat samples; S8 – S14: ground beef samples

Based on the results shown in Figure 2, it is shown that positive control (fresh beef) was amplified and the negative control (ddH_2O) was not amplified. This result indicates that PCR machine worked as expected eliminating any technical error possibility. Most samples of ground beef (S1-S7) and fresh beef (S8-S14) were successfully amplified. Bands were shown to be sharp and bright indicating high specificity of primers to target gene. However, below the bands, there were still smears. Appearance of smears can be caused by the quantity of Mg++, dNTP, *Taq Polymerase,* primer, and excess DNA *templates. Other possibility is the inclusion of contaminants on the DNA template which inhibit the activity of Taq polymerase* (Fatchiyah et al., 2011).

All amplified samples have the length of ~1140 bp in correspondence to Naidu et.al. (2012) who explained that the target gene, *cytochrome* b, has a sequence between 1140-1200 bp. It is then proven that 13 samples obtained from Pasar Pathuk and Pasar Kranggan contained meat derived from mammals. There was one fresh beef sample (S5) which was not amplified using MTCB primers. Considering that S5 has good A_{260}/A_{280} ratio of 1,987 and DNA concentration of 75.37 ng/µl, it is

possible that this sample did not contain any mammalian meat; neither beef nor pork. Nevertheless, this is an offense on Law Number (No). 8 of 1999 on Consumer Protection, specifically on Chapter IV, Verse 8, item *i*: *"not attaching labels or making descriptions of products containing the name of the products, size, net weight/content, composition, instruction to use, date of manufacture, side effects, names and addresses of business contact persons, as well as other information for use that according to the provision must be created."*

Samples were then further analyzed using PCR and P14 primers which target *PRE-1* as specific molecular marker for pork. As shown in Figure 3, only positive control (pork meat) was amplified at ~481 bp while all 14 samples were not amplified using P14 primers. This result indicates that P14 primers have high specificity on detecting pork meat. However, it is shown on Figure 3 that the bands were not of good quality even on the positive control. The poor quality of DNA bands can be caused by duration and voltage implemented on electrophoresis (Mustollah, 2016) and Tilawah, 2019).

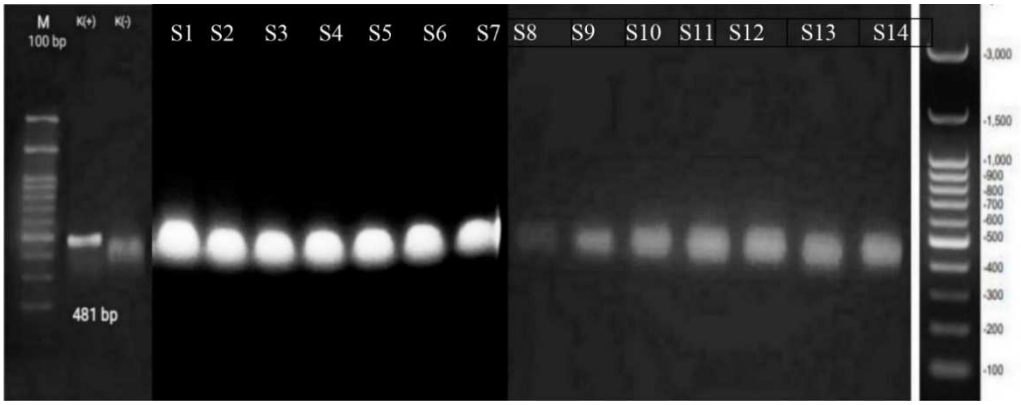

Figure 3. PCR amplification results using P14 primers electrified in 1.2% agarose gel; M : marker 100 bp; K(+): Positive Control (fresh beef); K(-) : Negative Control (ddH2O); S1 - S7 : fresh meat samples 1 - 7; S8 – S14 : sample ground meat 8 – 14

4. Conclusion

Based on the results, it is concluded that fresh and ground beef obtained from milling services in Pasar Pathuk and Pasar Kranggan Yogyakarta are not contaminated by pork DNA.

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