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Detection of Pork DNA on Commercially Processed Meat Products Using Taqman qPCR for Label Verification

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Abstract: Research has been conducted to detect pork DNA in various commercially processed meat products. This study aims to analyze the content of pork DNA in different commercially processed meat products sold online in Jakarta using a Taqman minor groove binder probe combined with a brief profile of a qPCR machine to verify labels. The sample used in this study consisted of 5 types of processed meat products: canned corned, jerky, shredded, meatballs, and smoked meat. A total of 30 samples had no pork labels, and the five others had pork labels. All products were extracted, the concentration and the purity of DNA were measured, and fragmentation DNA and amplification were analyzed using Taqman qPCR. The results showed that the DNA concentration obtained was 14.02 – 176.04 ng/μL, DNA purity was obtained from 1.70 to 1.92, and all genomic DNA was fragmented. There was no amplification in 30 samples without pork labels. Five samples of pork products underwent PCR amplification with a Ct value of 22.96, 20.72, 26.78, 21.96, and 23.16 for canned corned, jerky, shredded pork meatballs, and smoked pork, respectively. The conclusion is that commercially processed meat products sold online in Jakarta comply with labels regulation by Indonesia Food and Drug Authority and consistently maintain the halalness of their products. The novelties in this study tested various commercially processed meat products sold online in Jakarta.

Keywords: pork DNA, processed meat products, Taqman qPCR, label verification.

使用塔克曼定量聚合酶链反应检测商业加工肉类产品上的猪肉脱氧核糖核酸以进行标签验证

摘要：已进行研究以检测各种商业加工肉制品中的猪肉脱氧核糖核酸。本研究旨在分析在雅加达在线销售的不同商业加工肉类产品中猪肉脱氧核糖核酸的含量，使用塔克曼小沟粘合剂探针结合定量聚合酶链式反应机的简要概况来验证标签。本研究中使用的样本包括 5 种加工肉制品：罐装咸肉、肉干、肉丝、肉丸和熏肉。共有 30 个样品没有猪肉标签，另外 5 个样品有猪肉标签。提取所有产物，测定脱氧核糖核酸的浓度和纯度，利用塔克曼定量聚合酶链反应分析脱氧核糖核酸的片段化和扩增。结果表明，得到的脱氧核糖核酸浓度为 14.02-176.04 纳克/微升，脱氧核糖核酸纯度为 1.70-1.92，基因组脱氧核糖核酸全部片段化。没有猪肉标签的 30 个样本没有扩增。五个猪肉制品样品经过聚合酶链式反应扩增，对罐装咸肉、肉干、肉丝丸子和熏猪肉的 Ct 值分别为 22.96、20.72、26.78、21.96 和 23.16。结论是，在雅加达在线销售的商业加工肉类产品符合印度尼西亚食品和药物管理局的标签规定，并始终保持其产品的清真性。这项研究的新颖之处在于测试了在雅加达在线销售的各种商业加工肉类产品。

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关键词：猪肉脱氧核糖核酸、加工肉制品、塔克曼定量聚合酶链反应、标签验证。

1. Introduction

Processed meat products contain mammals, birds, or fish [1] and undergo further processing through salting, fermentation, drying, smoking, or other processes to improve flavor or preservation [2]. Processed meat products are concerned for counterfeiting practices because they relate to economic, religious, social, and health problems [3, 4]. The producer of processed meat products must declare originating pork or, in the process contact, or the process together with ingredients sourced from pork in their labels. It is written in a red box on a white basis and given images of a pig [5]. Surveillance of the origin of animal species is a challenge for government agencies, especially during the Covid-19 pandemic due to changes in people's lifestyles that have become entirely online.

The PCR technique is one of the essential analytical techniques to identify the origin of the animal species in food and feed [3, 6]. This technique is DNA-based, whereas DNA is more stable in the heat process than protein [7]. Therefore, the qPCR is more accurate, reliable, and sensitive than conventional PCR methods [8]. Several studies of detecting pork in processed meat products using qPCR in Indonesia have been carried out by [9-13], using Taqman [9, 10], Sybr green [11], and Evagreen [12, 13]. The Taqman method is the most suitable method for identifying meat species because it is more sensitive and specific than the dyes methods [14, 15].

The Taqman technology can be further improved in its specificity and sensitivity when combined with minor groove binder (MGB) molecules [16]. The Taqman MGB probe is a Taqman technology where at the 3' end were given a non-fluorescent quencher and MGB. Minor groove binder is a small molecule consisting of tripeptide dihydrocyclopyrroloindole tripeptide (DIP3) or 1,2 -dihydro- (3H)- pyrollo (3,2e) indole-7-carboxylate (CDPI).

The molecule forms non-covalent bonds with double-stranded DNA minor grooves that detect short genomic sequences [17]. Primer and Taqman MGB probe to detect pork was designed by [18] with a sensitivity up to 10 fg/µL. The speed analysis is also needed to optimize monitoring of suspected adulteration or contamination of pork in processed meat products. In [19], a brief profile in AB 7500 Fast real-time PCR (ABI) machine was used to detect pork DNA within 40 minutes.

Based on this background, this study aims to detect the presence of pork in commercially processed meat products sold online in Jakarta by combining the Taqman MGB probe and the qPCR brief profile. This research is expected to produce a sensitive, specific, and rapid detection of pork content in processed meat products to improve the monitoring performance of commercially processed meat products.

2. Materials and Methods

An overview of the methodology of this research is presented in Fig. 1.

2.1. Sample Collection

The sample used in this study consisted of 35 commercially processed meat products from 5 types of products, namely canned, corned, jerky, shredded, meatballs, and smoked meat. Each type of sample consisted of 7 different brands. Thirty products had not to pork labeled, and five had pork labeled. All products were obtained from online purchases through the Tokopedia marketplace. The sample purchased were in October and November 2021.

2.2. Positive Control

The positive control used in this study was fresh pork obtained from online purchases through the Tokopedia marketplace. The purpose used positive control to ensure that the primer and probe used in this study could amplify the material derived from pork and that the PCR reaction went well.

2.3. Negative Control

The negative control used in this study was nuclease-free water as a non-template control (NTC). Nuclease-free water was obtained from Promega. The purpose used positive control to determine whether there was contamination in the reaction or not.

2.4. DNA Extraction

All samples and positive control were extracted using the DNEasy Mericon Food kit (Qiagen, Germany). A total of 2 g sample was weighed. Subsequently, 25 µL proteinase K and 10 ml food lysis buffer were added. The solution was vortexed for 30 seconds and incubated at 60°C for 30 minutes by shaking on mode 15 (Genie, USA). Furthermore, the mixture was cooled at room temperature (25-27°C) for 10 minutes and kept in the refrigerator at 8°C for 10 minutes. It was also centrifuged at 2,500 x G for 10 minutes.

A total of 700 µL of the clear top solution was transferred to a 2 mL centrifuge tube, and 500 µL chloroform was added. It was vortexed for 30 seconds and centrifuged to 14,000 x G for 15 minutes. A total of

350 µL clear top solution was transferred to a 2 mL tube, and 350 µL PB buffer was added and vortexed for 30 seconds.

Subsequently, it was transferred to a spin column and centrifuged to 14,800 x G for 3 minutes. The tube was replaced with a new one then 700 µL buffer AW2 was added and centrifuged at 14,800 x G for 3 minutes. The column was transferred to a 2 mL centrifuge tube and centrifuged at 14,800 x G for 3 minutes. It was further transferred to a new 2 mL tube, and 100 µL buffer EB was added. The column was incubated at room temperature (25-27°C) for 5 minutes and centrifuged at 14,800 x G for 3 minutes. Furthermore, the DNA solution can be stored at -20°C when not used immediately.

2.5. Measurement of DNA Concentration and Purity

The concentration and purity of DNA isolated were measured using a Nanodrop One (Thermo Scientific, USA). Using the method as described by [20]. A total of 1.5 µL was DNA measured using the dsDNA module. The DNA concentration was calculated at the 260 nm wavelength, while DNA purity was estimated based on the absorbance ratio at 260 nm and 280 nm wavelength.

2.6. Genomic DNA Integrity Testing

The genomic DNA integrity was tested using the electrophoresis method according to the method described by [21] with slight modification. The DNA isolated electrophoresed on 1% gel agarose (Promega) in 1 x TAE buffer (Promega) stained with cyber safe 1µL/100 mL (Invitrogen).

A total of 10 µL of DNA mixed with 3 µL loading dye 6x (Promega) and used 5 µL DNA ladder (Promega) were electrophoresed at 100 V, 300 A for 30 minutes (Cleaver Scientific, UK). The DNA fragment was visualized using gel documentation (Biorad, USA) equipped with Image Lab software for image analysis.

2.7. Primer and Probe

The pork cytochrome b gene primer and Taqman MGB probe used in this study were designed by [18]. The sequences of the primer dan Taqman MGB probe showed in table 1. The primer and Taqman MGB probe were obtained from Applied Biosystem, USA.

Table 1 Sequences of primer and Taqman MGB probe [18]

	Sequences
Primer forward	5'-CTTGCAAATCCTAACAGGCCTG-3'
Primer reverse	5'-CGTTTGCATGTAGATAGCGAATAAC-3'
Taqman MGB Probe	5'-(FAM)-ACAGCTTTCTCATCAGTTAC-(NFQ)(MGB)-3'

2.8. Amplification of qPCR

The volume reagent used for qPCR amplification was 25 µL consisting of a 22.5 µL master mix and a 2.5 µL DNA template. The master mix reagent consisted of 0.75 µL of 10 µM forward primer, 0.75 µL of 10 µM reverse primer, 0.5 µL of 10 µM probe, 12.5 µL of Taqman fast universal master mix, and 8 µL of nuclease-free water**.** The master mix reagent was transferred into a PCR plate and added with a DNA template. The reaction was completed with positive control and NTC. The qPCR was carried out using AB 7500 Fast (ABI, USA) using a fast profile, consisting of denaturation at 95°C for 20 seconds, annealing at 95°C for 3 seconds, followed by 45 cycles, and extension at 60°C for 30 seconds. The 7500 software version 2.3 is used to process and collect data.

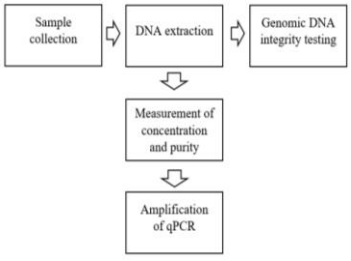

Fig. 1 Research methodology overview

2.9. Data Analysis

The data was analyzed by looking at the Ct value of qPCR. The Ct value indicated the presence of pork DNA fragments in the sample; while the Ct value did not appear, it stated the absence of pork DNA fragments in the product.

3. Results

3.1. Results of DNA Isolated

The results of DNA isolation are shown in table 2. The measurement of DNA concentration and purity used Nanodrop One. Based on table 2, it is known that DNA concentration obtained 14.02–176.04 ng/µL with an average of 55.22 ng/µL. The range of DNA concentration based on the type of products is 14.02–37.52 ng/µL, 31.17-86.87 ng/µL, 15.46–62.80 ng/µL, 39.28–102.63 ng/µL, 29.79–176.04 ng/µL with an average of 27.19 ng/µL 51.35 ng/µL, 24.77 ng/µL, 82.44 ng/µL and 90.36 ng/µL for canned cornet, jerky, shredded, meatballs and the smoked meat respectively.

Measurement purity DNA based on the absorbance ratio at 260 nm and 280 nm showed a value range of

1.70–1.92 with an average of 1.83. Based on the product type, the purity DNA range is 1.70-1.89, 1.75-1.91, 1.72–1.91, 1.82–1.92, 1.71-1.91 with an average of 1.75, 1.85, 1.83, 1.86 1.84 for canned corned, jerky, shredded, meatballs and the smoked meat, respectively.

Table 2 Results of DNA isolation

Sample Information				**DNA**	
No	**Name**	**Origin**	**Label**	**Concentration**	**Purity**
1	Canned Corned 1	Indonesia	Halal	37,52	1,71
2	Canned Corned 2	Brazil	-	33,62	1,75
3	Canned Corned 3	Papua New guinea	-	14,02	1,74
4	Canned Corned 4	Australia	-	34,89	1,70
5	Canned Corned 5	Indonesia	Halal	16,07	1,71
6	Canned Corned 6	Indonesia	Halal	28,98	1,75
	Canned Corned (A)	Indonesia	Pork	25,22	1,89
7	Jerky 1	Indonesia	Halal	31,17	1,80
8	Jerky 2	Indonesia	Halal	34,13	1,75
9	Jerky 3	Indonesia	Halal	55,27	1,91
10	Jerky 4	Indonesia	-	50,25	1,84
11	Jerky 5	Indonesia	Halal	54,66	1,87
12	Jerky 6	Indonesia	-	86,87	1,86
	Jerky (B)	Indonesia	Pork	47,11	1,91
13	Shredded 1	Indonesia	Halal	25,90	1,72
14	Shredded 2	Indonesia	Halal	15,46	1,78
15	Shredded 3	Indonesia	Halal	16,65	1,81
16	Shredded 4	Indonesia	-	18,21	1,78
17	Shredded 5	Indonesia	Halal	17,99	1,89
18	Shredded 6	Indonesia	Halal	16,40	1,91
	Shredded (C)	Indonesia	Pork	62,80	1,89
19	Meatballs 1	Indonesia	Halal	73,33	1,83
20	Meatballs 2	Indonesia	Halal	94,64	1,90
21	Meatballs 3	Indonesia	Halal	71,07	1,84
22	Meatballs 4	Indonesia	Halal	97,56	1,87
23	Meatballs 5	Indonesia	Halal	39,28	1,92
24	Meatballs 6	Indonesia	Halal	102,63	1,87
	Meatballs (D)	Indonesia	Halal	98,58	1,82
25	Smoked meat 1	Indonesia	Halal	176,04	1,84
26	Smoked meat 2	Indonesia	Halal	50,69	1,91
27	Smoked meat 3	Indonesia	Halal	126,01	1,83
28	Smoked meat 4	Indonesia	Halal	29,79	1,83
29	Smoked meat 5	Indonesia	Halal	56,56	1,87
30	Smoked meat 6	Indonesia	Halal	130,43	1,86

Continuation of Table 2

Smoked meat (E)	Indonesia	Pork	63,03	1,71
Positive control (P)	Indonesia	Pork	34,01	1,88

Note: The value of concentration and purity is the average of two measurements

The DNA is pure if they have a value absorbance ratio from 1.8 to 2.0 [22], while [23] reported the DNA is pure in a range of 1.7–2.0, which indicates successful removal of contaminating molecules [24]. The concentration of DNA obtained was more than 10 ng/µL, so the DNA obtained was sufficient for amplification because this study used a DNA template of 10 ng/µL. Differences in concentration and purity of DNA obtained are caused by differences in the composition of the materials used and differences in the sample processing.

3.2. Genomic DNA Integrity

The integrity of genomic DNA was visualized using electrophoresis to ensure that DNA could be adequately isolated. The results of the visualization of genomic DNA are shown in fig. 1. Based on fig. 1, it can be seen that the DNA of the positive control was integrated, while the others were fragmented. However, the band intensity of positive control was not as bright as in some samples. A lower concentration than the others may cause it. The DNA bands hanging down or smears indicate DNA degradation has occurred. Samples A, B, C, and 15 result in a faint or invisible DNA band.

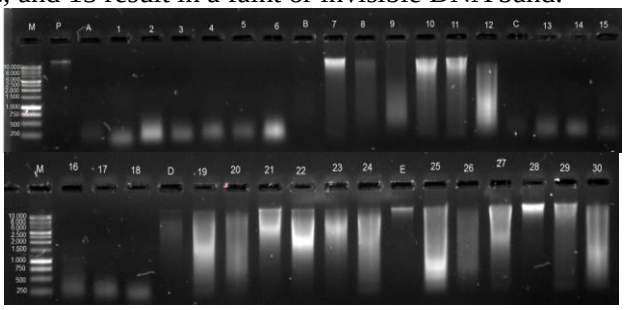

Fig. 2 Genomic DNA electrophoresis results. DNA marker ladder 1 kb (M), positive control (P), canned meat corned (1-6), canned pork corned (A), meat jerky (7-12), pork jerky (B), meat shredded (13-18), pork shredded (C), meatballs (14-24), pork meatballs (D), smoked meat (25-30), smoked pork (E)

Different DNA band patterns described the differences in DNA fragmentation. In addition, the processing and the type of sample ingredients affected the DNA fragmentation and the intensity of the bands produced.

3.3. Analysis of qPCR

The presence of the pork cytochrome b gene in the samples was analyzed using qPCR. The results can be seen in Fig. 2. The qPCR amplification curve showed the presence of pork DNA fragments in the sample. Based on Fig. 2, the amplification curve occurred in the positive control (red), canned pork cornet (turquoise), pork jerky (purple), pork shredded (blue), and pork meatballs (green), and smoked pork (black). The Ct value achieved 19.30, 22.96, 20.72, 26.78, 21.96, and 23.16 for positive control, canned pork cornet, pork jerky, pork shredded, pork meatballs, and smoked pork, respectively. None of the commercially processed meat products without pork labels and NTC have an amplification curve. The absence of amplification in NTC indicated no contamination in the PCR reaction. The results showed that all processed meat products without pork labels contained no pork DNA fragments.

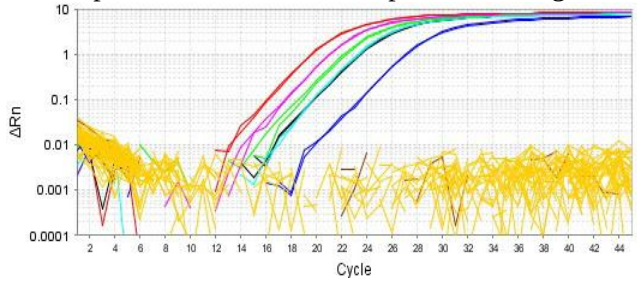

Fig. 3 The qPCR amplification curve. Positive control (red), canned corned pork (turquoise), pork jerky (purple), pork shredded (blue), pork meatballs (green), smoked pork (black), non-template control (brown), sample no. 1-30 (yellow)

4. Discussion

This study detects pork DNA in various commercially processed meat products sold online in Jakarta using the Taqman MGB probe combined with a brief profile of the qPCR machine. With this method, the testing can be more specific, sensitive, and rapid. Some studies on the detection of pork content in commercially processed meat products circulating in the community are well labeled. Well-labeling is essential because people need to know that their ingredients are safe and follow religious law.

This study designed primer and Taqman MGB probe following [18] to make the results more sensitive and specific [16]. The presence of MGB supports the entry of aromatic rings due to Van der Walls and electrostatics interactions. These interactions result in minimal distortion of the phosphodiester backbone but stabilize the DNA structure. The highly stable interaction between MGB and target DNA increases the melting temperature of the probe and prevents non-specific amplification. Furthermore, a non-fluorescence quencher (NFQ) at the 3' end of the Taqman probe can reduce the background fluorescence. So this method allows the use of a short probe to detect short genomic sequences [17].

[12] detecting pork DNA in fresh pork and canned corned meat using Evagreen qPCR got Ct values of

21.05 and 29.01, while the results of this study obtained the Ct values 19.30 and 22.96 for fresh pork and canned corned meat, respectively. The earlier Ct value obtained can be concluded that the Taqman probe-based is more sensitive than the Evagreen-based method. Canned corned meat is a processed meat product that involves heat and pressure. The product suffers DNA damage which is quite severe, and this is indicated by the occurrence of DNA fragmentation as shown in genomic DNA electrophoresis. However, the technology of the Taqman MGB probe can detect the presence of pork DNA with a Ct value that is not too high (22.96).

The research using a combination of Taqman MGB probe and a brief qPCR machine limited [18] did not use the fast profile, so it took longer testing. In [19] it was used a brief profile combined with Evagreen qPCR so the testing time could be fast, 40 minutes. Combining the Taqman MGB probe and fast profile qPCR is very promising for label verification in Indonesia.

Based on the test results, all samples without pork labels did not contain pork DNA, either with the halal label or not. The products that do not have pork DNA without a halal label cannot state as halal products because the halal is based not only on the absence of the pork content but also on many factors that need to be considered. Manufacturers of commercially processed meat products were consistent in implementing Indonesian FDA regulations regarding the inclusion of labels in processed foods and consistently maintaining the halalness of their products.

Similar results were obtained by [11,13]. Their studies showed no pork detection in meatballs [11] and beef jerky [13]. The meatballs were sold in Tanjung Priok [11], while the beef jerky was obtained from Yogyakarta [13]. [11] used the same primer with this study but replaced the probe with Sybr Green.

The novelties in this research were tested in various commercially processed meat products sold online in Jakarta.

5. Conclusion

Detection of pork DNA in commercially processed meat products sold online In Jakarta using Taqman MGB probe combined with a brief profile of the qPCR machine is more sensitive, specific, and rapid. Commercially processed meat products sold online in Jakarta have complied with the Indonesian FDA regulation about food processed labels, and the products' halalness was maintained. These results indicate the government's success in supervising the processed meat products circulating. The limitation of this research was only using five types of commercially processed meat products and expensive pairs of primer probes. It is hoped that a similar study can be carried out to identify other species such as wild boars or rats that can provide economic benefits for irresponsible parties.

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