The Sensitivity Evaluation of *mt-DNA* Genes; *NADH Dehydrogenase Sub Unit 5* (*ND5*), *D-Loop*, and *Cytochrome-b* (*Cty-b*) to Detect Pork (*Sus scrofa*) DNA Isolate and DNA Fragment in Meatball using PCR Technique

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Abstract: The utilization of *mt-DNA* primers on previous study specific to detect DNA pork fragments. This study aims were to evaluate the sensitivity of *mt-DNA* primers (*ND5*, *D-Loop*, and *Cyt-b*) in pork DNA isolates and its meatballs product. The sensitivity analysis was conducted in pork DNA isolates with concentrations (10, 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} ng/µl) and its meatballs product with variation of pork content (0%, 0.01%, 0.05%, 0.1%, 0.5%, 1%, and 5%). Furthermore, the DNA fragment amplification process was carried out using PCR technique. The amplification results showed that *ND5* and *Cyt-b* primers were more sensitive because they were able to amplify DNA with concentrations up to 10^{-3} ng/µl compared to *D-Loop* which was only able to amplify with concentrations up to 10^{-2} ng/µl. The sensitivity results using meatballs showed that *ND5* was the most sensitive primer in detecting meatballs with concentration of pork up to 0.01%. It can be concluded that *ND5*, *Cyt-b*, and *D-Loop* primers are able to detect pork DNA fragment with high sensitivity. The *ND5* primer gave the most sensitive amplification results because it was able to detect pork DNA fragments with the lowest concentration and meatball with the lowest pork content.

1 INTRODUCTION

There were 1,300 cases of food adulteration between 1980 till 2010 (Moore et al., 2012). Some food falsification case in various regions in Indonesia are using pork in resembles beef and applying pork in the process making of beef meatballs (Bempah, 2017; Harianmerapi, 2018; Nuryanti, 2020). Meatballs are food product that often being falsification in the purpose of economic gain in the anticipation of high price of beef. Food falsification is defined as an attempt to replace, imitate, increase, change, or misrepresent a food product, food packaging, and fraudulent label information for economic gain purposes (Hariyadi, 2015).

Identification and prevention of falsification food is important to protect consumers and prevent unhealthy competition for food producers especially processed meat products. Therefore, we need a detection method that simple and fast for everyday application (Kesmen et al., 2010). In the analysis of meatball samples, the meat has undergone high temperature processing and treatment. Denaturation of meat protein during heating treatment or some specific technology applications in the processing stage may decrease the success of the analytical method. Therefore, the method is no longer able to distinguish the species that related closely and unsuitable for use as an everyday analysis, such as species-specific detection becomes difficult and long time (Hofman, 1987; Jemmi and Schlosser, 1992; Koh et al., 1998; Kesmen et al., 2010).

DNA hybridization and PCR methods have been widely used for the identification of meat processed in meat products (Fei et al., 1996; Matsunaga et al., 1999). Polymerase Chain Reaction (PCR) technique is widely applied for the analysis of processed meat-based food products because it is fast, simple, specific, and sensitive (Matsunaga et al., 1999; Kesmen et al., 2007; Haunshi et al., 2008; Yahya et al., 2017). PCR technique is often used as a technique for fast detection of pork content using

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mt-DNA primers in the amplification process. Mitochondrial DNA (*mt-DNA*) is DNA derived from mitochondrial organelles with nucleotide that similar to its parent and lots present in cells (Felk et al., 2017). Species-specific primers are designed based on the *mt-DNA* sequences of various animal species used in species identification.

The study related to the primer species *mt-DNA*, namely *NADH dehydrogenase subunit 5* (*ND5*) with a target sequence of 227 bp has been used in skeletal muscle tissue samples from pigs and applied to detect pork content in typical turkey meat products, namely Sucuk (Kesmen et al., 2010). Research on the use of *mt-DNA Cyt-b* species primers used the multiplex PCR method to detect the content of pork which pork had undergone heating treatment at temperatures of 100°C and 120°C (Matsunaga et al., 1999). Previous research, *mt-DNA* primers (*ND5*, *D-Loop*, and *Cyt-b*) using conventional PCR techniques applied to beef, goat, pork, lamb, and chicken have been successfully applied to detect specific pork content. In this study, *mt-DNA* primers (*ND5*, *D-Loop*, and *Cyt-b*) were used to evaluate the primary sensitivity to various variations of pork DNA concentrations and the content of pork DNA fragments in meatballs. This research was conducted to support the *mt-DNA* primers specificity data (*ND5*, *D-Loop*, and *Cyt-b*) in detecting the content of pig DNA fragments using conventional PCR techniques and for further research in detecting cases of counterfeiting food processed products.

2 RESEARCH METHODS

The sensitivity primer test was carried out based on variations in the DNA concentration of pork and pork meatballs. The sample of pork DNA isolates had been obtained and analyzed quantitatively using Nanodrop (spectrophotometer) produced a DNA concentration of 132.21 ng/µl and DNA purity of 1.98 (Kusnadi et al., 2019). Pork DNA isolation samples were diluted into several concentrations, namely 10 ng/µl, 1 ng/µl, 10^{-1} ng/µl, 10^{-2} ng/µl, 10^{-3} ng/µl, 10^{-4} ng/µl, 10^{-5} ng/µl, and 10^{-6} ng/µl. In addition, pork meatballs are made with a mixture consisting of spices (garlic, onion), tapioca flour, monosodium glutamate, ice cubes, and STTP (Sodium tripolipospat). The proportion of each dough ingredient is shown on Table 1. The producing of meatballs were mix all ingredients based on predetermined proportions. The meatball dough was boiled in water with a temperature of 80°C for 15 minutes until the meatball expands and is cooked. The meatballs were drained and boiled again at 100°C for 5 minutes, removed, and drained. Furthermore, pork meatballs with various pork content of 0% (negative control), 0.01%, 0.05%, 0.1%, 0.5%, 1%, and 5% (w/w) were DNA isolated using the alkaline-lysis method with modified procedures.

PCR reactions consist of Go Taq Green Master Mix (Promega), BSA (Bovine Serum Albumine), Forward and reverse *mt-DNA* Primers (*ND5*, *D-Loop*, and *Cyt-b*), pig DNA with various concentrations and DNA pork meatballs. The PCR program consisted of hot start at 95°C for 5 minutes, denaturation at 95°C for 1 minute, annealing at 54°C for 1 minute, extension at 72°C for 1 minute, and post extension at 72°C for 7 minutes. There were 3 *mt-DNA* primers used, namely *ND5*, *D-Loop*, and *Cyt-b* with target DNA sequence lengths, namely 227 bp, 835 bp, and 398 bp (Table 2). The electrophoresis process was used 1.5% agarose gel, loading dye, 1x TBE, EtBr (Ethidium Bromide), and 1 Kb of DNA ladder. Electrophoresis results were visualized using gel doc imaging.

Table 1: Meatball ingredient formulation with pork substitution in beef.

Raw Material	Concentration of Beef Substitution with Pork						
	0%	0.01%	0.05%	0.1%	0.5%	1%	5%
Beef (gr)	40	39.99	39.98	39.96	39.8	39.6	38
Pork (gr)	-	0.004	0.02	0.04	0.2	0.4	2
Instant flour for meatballs (gr)	16	16	16	16	16	16	16
Ice Cube (gr)	3.2	3.2	3.2	3.2	3.2	3.2	3.2
STTP (gr)	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Total (gr)	60						

Table 2: Primers used in PCR stages.

Primer	Sequence Position (5'- 3')	DNA Length (bp)
ND5 (Kesmen et al., 2007)	*F: 5'*-CAT TCG CCT CAC TCA CAT TAA CC-3' *R: 5'*-AAG AGA GAG TTC TAC GGT CTG TAG-3'	227
D-loop (Haunshi et al., 2008)	*F:5'*-TAC TTC AGG ACC ATC TCA CC-3' *R:5'*-TAT TCA GAT TGT GGG CGT AT-3'	835
Cyt-b (Matsunaga et al., 1999)	*F:5'*-GAC CTC CCA GCT CCA TCA AAC ATC TCA TCT TGA TGA AA-3' *R: 5'*-GCT GAT AGT AGA TTT GTG ATG ACC GTA-3'	398

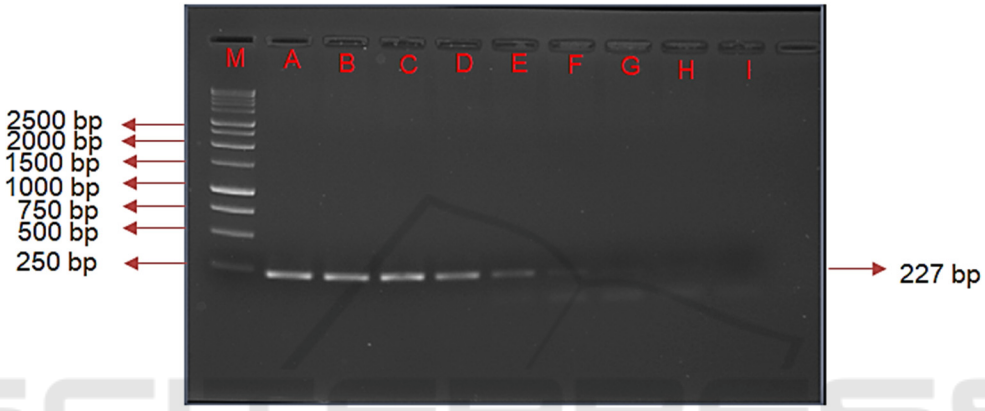

Figure 1: Sensitivity Test using *ND5* Primer in Various Pork DNA. Note: M: DNA ladder 1000 bp, A: initial concentration (132,05 ng/µl), B: 10 ng/µl, C: 1 ng/µl, D: 10^{-1} ng/µl, E: 10^{-2} ng/µl, F: 10^{-3} ng/µl, G: 10^{-4} ng/µl, H: 10^{-5} ng/µl, and I: 10^{-6} ng/µl.

3 RESULTS

3.1 Sensitivity Test of Primer with Various Pork DNA Concentrations

The sensitivity analysis aims to determine the primer ability to detect fragment DNA with the smallest concentration. In this study, the three species-specific *mt-DNA* primers (*ND5*, *D-Loop*, and *Cyt-b*) were tested at various concentrations of isolated pork DNA. Previous research results showed that pork DNA isolates were 132.05 ng/µl with a purity of 1.98 (Kusnadi et al., 2020). The pork DNA isolates has been used with concentration of 132.05 ng/µl and purity of 1.98 produced in previous research (Kusnadi et al., 2020). The amplification results of the three *mt-DNA* primers on pork DNA isolates showed that the lower the DNA concentration used in the amplification, the thinner the DNA bands produced (Figures 1, 2, and 3).

Visualization of amplification results using primers *ND5*, *D-loop*, and *Cyt-b* at various concentrations of pork were showed that *ND5* primers were able to amplify DNA at DNA concentrations of 10 ng/µl, 1 ng/µl, 10^{-1} ng/µl, 10^{-2} ng/µl, and 10^{-3} ng/µl. However, the amplification results using the *ND5* primer appeared to be thinner than the DNA band amplified using the *Cyt-b* primer (Figure 1). *Cyt-b* primer was able to amplify DNA with concentration of 10 ng/µl, 1 ng/µl, 10^{-1} ng/µl, 10^{-2} ng/µl, and 10^{-3} ng/µl constructs with clearer results than *D-Loop* and *ND5* primers (Figure 3). *D-Loop* primer was lowest sensitivity to amplify of DNA samples with concentrations of 10 ng/µl, 1 ng/µl, 10^{-1} ng/µl, 10^{-2} ng/µl (Figure 2).

The results of the primer sensitivity analysis have been carried out before, *ND5* primer sensitivity was able to detect DNA concentrations of 10^{-2} ng/µl (Kesmen et al., 2007), while *Cyt-b* primer was able to detect DNA concentrations of 0.25 ng/µl (Matsunaga et al., 1999), and *D-Loop* primer has not been studies. Primers are one of the components that determine the success of the PCR technique. Primer specificity and sensitivity analysis are very necessary to support primary applications to detect contamination in food processed products.

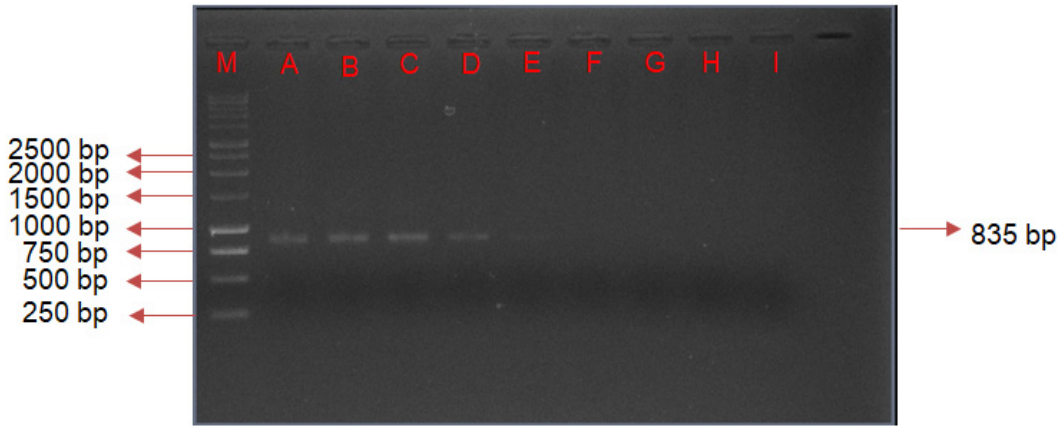

Figure 2: Sensitivity Test using *D-Loop* Primer in Various Pork DNA. Note: M: DNA ladder, A: initial concentration (132,05 ng/µl), B: 10 ng/µl, C: 1 ng/µl, D: 10^{-1} ng/µl, E: 10^{-2} ng/µl, F: 10^{-3} ng/µl, G: 10^{-4} ng/µl, H: 10^{-5} ng/µl, dan I: 10^{-6} ng/µl.

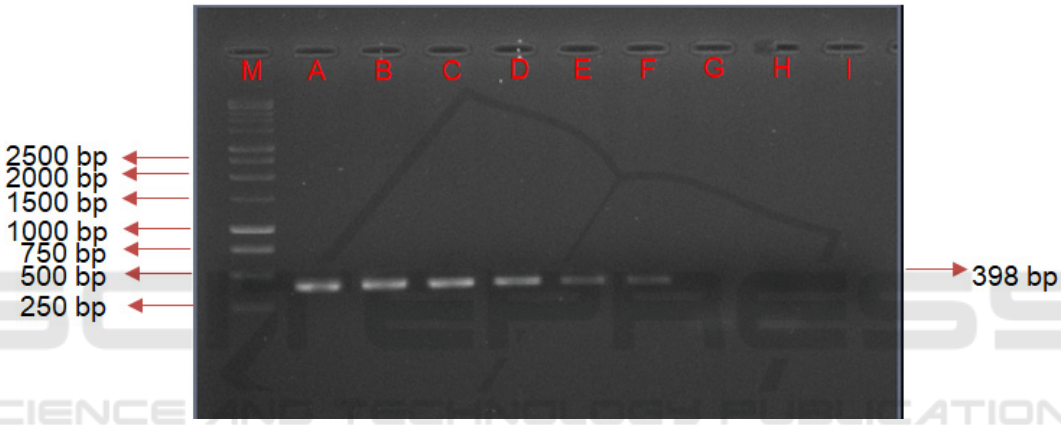

Figure 3: Sensitivity Test using *Cyt-b* Primer in Various Pork DNA. Note: M: DNA ladder, A: initial concentration (132,05 ng/µl), B: 10 ng/µl, C: 1 ng/µl, D: 10^{-1} ng/µl, E: 10^{-2} ng/µl, F: 10^{-3} ng/µl, G: 10^{-4} ng/µl, H: 10^{-5} ng/µl, dan I: 10^{-6} ng/µl.

Tabel 3: Concentration and Purity of Pork Meatball DNA Isolate.

Sample	Purity (λ 260/280)			Average	Concentration (ng/µl)			Average
	Replicate				Replicate			
	1	2	3		1	2	3	
0%	2.12	1.65	1.89	1.89	55.26	142.95	133.05	110.42
0.01%	1.68	1.64	2.01	1.78	26.06	275.82	141.07	147.65
0.05%	2.30	1.92	1.90	2.04	46.75	151.33	90.21	96.0967
0.1%	1.87	1.99	1.93	1.93	63.02	154.03	85.68	100.91
0.5%	2.27	1.87	2.02	2.05	72.90	96.79	128.68	99.4567
1%	2.19	1.86	1.93	1.99	61.85	106.83	83.28	83.9867
5%	2.39	1.87	2.06	2.10	64.60	133.34	144.37	114.103

3.2 Sensitivity Test of Primer in Pork Meatball Samples

The results of DNA isolation of pork meatballs with concentrations of pork content of 1%, 0.01%, 0.05%, 0.1%, 0.5%, 1%, and 5% with 3 replications each resulted in an average concentration of 83.99 to 147.65 ng/µl and the mean purity of 1.78 to 2.10 (Table 3). Based on the DNA concentration that has been obtained, it shows that the isolation of pork

meatballs using the alkaline-lysis method produces high DNA concentrations, however, some samples still contain RNA and protein contaminants.

Meatball is a processed meat product that has undergone high temperature treatment and the addition of various other ingredients. Other ingredients added to the meatball making, namely flour, STTP, and salt are added to represent meatball products on the market. The potential for meatballs to experience more contamination will be higher and will result in lowering the concentration of DNA produced. Based on the results of DNA isolation, it shows that the alkaline-lysis method is effective for use as a method for isolating pork meatball DNA, a slight modification with the addition of Pro-K and RNAse is needed to reduce protein and RNA contamination.

The results of DNA isolation of pork meatballs with pork content of 5%, 1%, 0.5%, and 0.1% were amplified using three *mt-DNA* primers *ND5*, *D-Loop*, and *Cyt-b* primers which were able to produce DNA bands. *D-Loop* primer is able to amplify pork meatballs with pork content of 5% to 0.1% with different appearance levels, pork meatballs with a concentration of 5% and 1% can appear for 2 repetitions, while concentrations of 0.05% and 0.01% appears 1 time. The results of amplification of pork meatballs with a concentration of 5%, 1%, 0.5%, and 0.1% using *Cyt-b* primer produced DNA bands with 2 appearances. The amplification results using *D-Loop* primer was able to amplify pork meatball samples at concentrations of 5%, 1%, 0.5%, and 0.1%, however, the resulting DNA bands were not as good as the amplification results using *Cyt-b* primer. The results of DNA isolation of pork meatballs using *ND5* primers were able to amplify DNA up to a concentration of 5%, 1%, 0.5%, 0.1%, and 0.01% with an appearance rate of 2 to 3 times. Based on the results of the primary sensitivity test on meatball samples, the *ND5* primer was the most sensitive compared to *Cyt-b* and D-*Loop,* it was shown that the primer was able to amplify meatball samples with the lowest pork concentration of 0.01%. Pork samples have undergone a processing process (milling and heating) and it is possible to experience degradation which can reduce the quality of the sample DNA. The results showed that the meatball sample with the lowest concentration of pork DNA content of 0.01% could be amplified using *ND5* primer. The amplification results using *ND5* primer produced 227 bp of target DNA strands. Compared to *D-Loop* and *Cyt-b* primers, *ND5* primer produced the shortest length of the target DNA sequence. The results showed that primers with a short target DNA length such as *ND5* with 227 bp length to detect specific species in preheated foods gave the most sensitive results. The results of amplification of pork meatballs with *D-Loop* primer were able to detect the content of pork meatballs with a concentration of 5% to 0.1% in meatballs. The *D-Loop* primer has a target DNA sequence length of 835 bp (Figure 5). Amplification using *D-Loop* primers results in lower sensitivity compared to *ND5* primers.

The amplification results using *Cyt-b* primer showed that the primer was able to detect the content of pork with concentration of 5% to 0.1% in meatballs. The *Cyt-b* primer has a target DNA sequence with length of 398 bp. Based on the results of the sensitivity analysis, it shows that *D-Loop* and *Cyt-b* primers are sensitive in detecting pork content

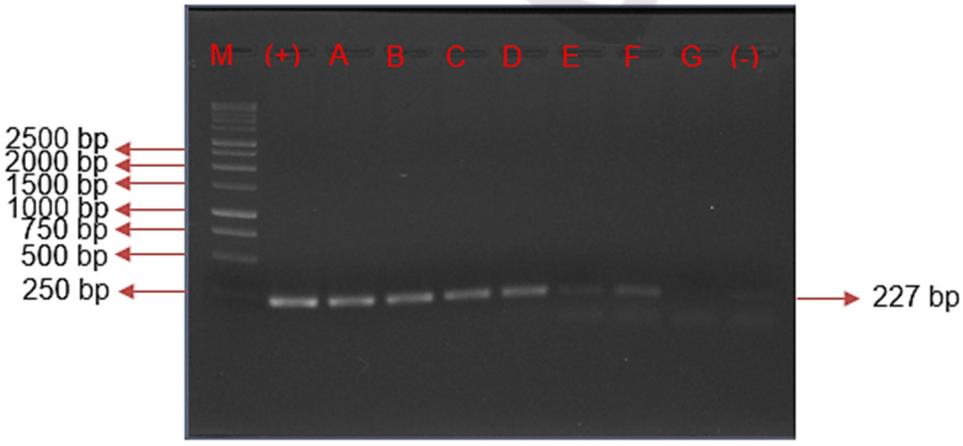

Figure 4: Sensitivity Test Using *ND5* Primer in Pork Meatballs. Note: A. Pork Meat Concentration 5%, B. Pork Meat Concentration 1%, C. Pork Concentration 0.5%, D. Pork Concentration 0.1%, E. Pork Meat Concentration 0.05%, F. Pork Concentration 0.01%, G. Pork Concentration 0%, (-) Negative Control, (+) Positive Control, M. DNA Ladder.

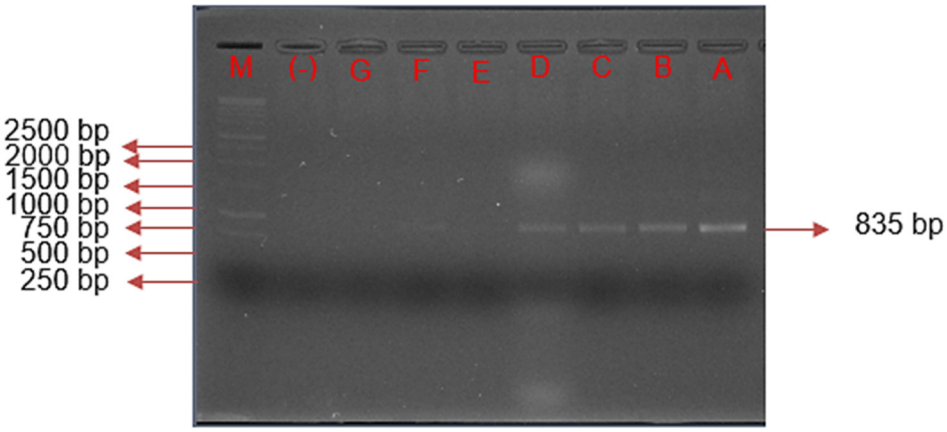

Figure 5: Sensitivity Test using *D-Loop* Primer in Pork Meatballs. Note: A. Pork Concentration 5%, B. Pork Meat Concentration 1%, C. Pork Concentration 0.5%, D. Pork Concentration 0.1%, E. Pork Meat Concentration 0.05%, F. Pork Concentration 0.1%, G. Pork Concentration 0%, (-) Negative Control, (+) Positive Control, M. DNA Ladder.

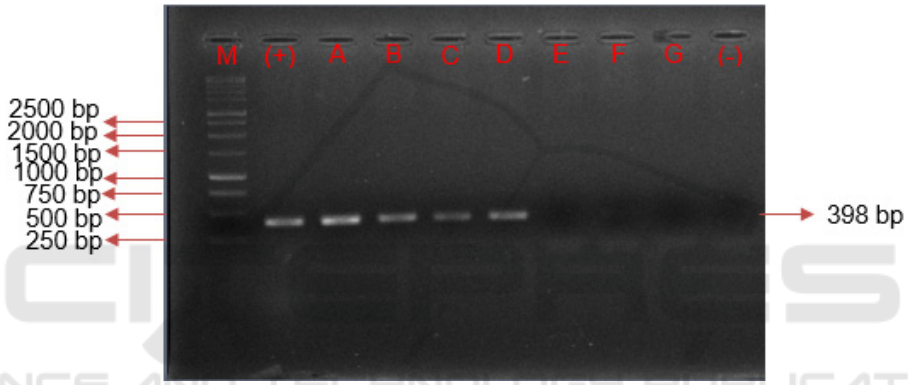

Figure 6: Sensitivity Test Using *Cyt-b* Primer in Pork Meatballs. Note: A. Pork Concentration 5%, B. Pork Meat Concentration 1%, C. Pork Meat Concentration 0.5%, D. Pork Meat Concentration 0.1%, E. Pork Meat Concentration 0.05%, F. Pork Concentration 0.1%, G. Pork Concentration 0%, (-) Negative Control, (+) Positive Control, M. DNA Ladder.

with concentration of rok content 5% to 0.1% in meatballs. The overall results of primer sensitivity analysis showed that the *ND5* primer was the most sensitive in detecting pork content in meatballs compared to *D-Loop* and *Cyt-b* primers. *ND5* primers had the shortest target gene with sequence length of 227 bp compared to both *D-Loop* and *Cyt-b* primers, this was thought to cause the primer to have the highest sensitivity.

In this study, the results of the *ND5* primer sensitivity test showed the most sensitive primer was able to detect up to 0.01% pork concentration in meatballs. Previous research results of *ND5* primer had low sensitivity in detecting pork content, namely a concentration of 0.1% in sausage samples (Kesmen, 2007). Meanwhile, in another study, the *ND5* primer had low sensitivity and was able to detect the content of pork with a concentration of 0.1% in a sample of a mixture of chicken meat and lard (Felk et al., 2017). In this study, *Cyt-b* primer had lower sensitivity compared to *ND5* primer. Another study, *Cyt-b* primer had low sensitivity in detecting pork with a concentration of 1% in a mixture of cooked pork and beef.

The high sensitivity of the *ND5* primer can be caused by the reason that the orimer has a short DNA sequence target so that it is sensitive in detecting meatball samples. PCR testing uses a primer with a short amplicon target which aims to detect specific species in foods given heat treatment, is better and more stable than using primers with a long amplicon target (Ali et al., 2016). This is supported by Rahman et al (2014), that primers with short amplicon targets can be amplified with samples processed by high pressure autoclave. Yoshida et al. (2009) who tested primers with a target of 126 bp and 83 bp with their samples given heating and pressure treatments, also showed that primers with shorter amplicon targets were more sensitive for detecting DNA fragments.

The presence of a mixture of flour, spices, salt, and STTP can potentially be a contaminant in DNA isolates. The presence of these contaminants can interfere with the success of the PCR process and are not repeatable. The results showed that the occurrence rate of DNA bands from 3 repetitions varied. The high content of polysaccharides in isolates originating from flour, contamination of organic compounds such as polysaccharides may interfere with the enzymatic process of DNA polymerase by mimicking the structure of nucleic acids (Schrader et al., 2012). According to Bergallo et al (2006), there are several ways to remove these contaminants, for example, such as giving Tween 20 to remove polysaccharides.

4 CONCLUSION

The *mt-DNA ND5* and *Cyt-b* primers were more sensitive because they were able to amplify DNA up to concentration of 10^{-3} ng/µl compared to *D-Loop* primer which were only able to amplify at concentrations up to 10^{-2} ng/µl. In addition, *ND5* primer was the most sensitive to detect meatballs with pork content up to 0.01%. Thus, the three *mt-DNA ND5*, Cyt-b, and *D-Loop* primers were able to detect specific and sensitive pork DNA fragments. The *ND5* primer gave the most sensitive amplification results because it was able to detect pig DNA fragments with the lowest concentration of 10^{-3} ng/µl and meatballs with the lowest pork content of 0.01%.

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