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Direct PCR-Based Detection of Pork Adulteration in Food Products Using ND4 and Cytb Markers

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ABSTRACT

The adulteration of edible processed meat has become widespread, as low-quality meat is added. One of the most prevalent instances is the use of an adulterated meat product containing pork, which caused problems for Muslims because of their dietary restrictions. Food safety needs a quick, easy, and specific DNA-based detection method. Therefore, the present study sought to detect pork contamination utilising the direct-PCR method with NADH dehydrogenase 4 (ND4) and cytochrome b (Cyt-b) primers in fresh pork and commercially processed meat products. The lysis buffer recipe was optimised using two temperatures and three incubation periods. Conventional PCR and electrophoresis were used to confirm DNA detection. Three brands of meatballs, sausages, and corned beef obtained from several markets in Malang City, Indonesia, were examined following optimisation. With high levels of DNA concentration and purity as well as visible DNA bands, the incubation temperature of 95°C for 5 minutes clearly exhibited the best results. All ND4 and Cyt-b primers amplified pork DNA in the majority of the processed samples, except for some Cyt-b primers, which did not amplify DNA in certain corned beef products. The direct-PCR technique is clearly a straightforward, fast, inexpensive, and reliable method for the detection of pork adulteration in processed meat products and could also be applied for halal food authentication.

Keywords: Adulteration, direct PCR, ND4, Cyt-b, pork detection

INTRODUCTION

Food adulteration is the modification of food products by the addition of inferior or unauthorised materials. This case is a serious issue in the food industry, especially in the meat industry (El Sheikha et al., 2017). Many meat products are often economically mixed with lower-priced, poorer-quality, and unsafe meats to increase profitability (Valand et al., 2020). Pork is frequently adulterated due to its muscle texture and fibre arrangement, which appear similar

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to those of beef (Yang et al., 2018; Li et al., 2023). Adulteration of pork is prohibited by Islamic law regarding the halalness of food in a product (Song et al., 2019).

To determine the presence of pork in food products and thus the halalness of a product, a detection method is required, one of which is a DNA-based method employing the PCR or Polymerase Chain Reaction method. PCR is a type of enzymatic synthesis that amplifies particular DNA fragments that will become the target DNA. The PCR method is frequently used because it is direct, has high sensitivity and specificity, and is inexpensive (Alikord et al., 2018). The primer is a key aspect in the success of the PCR process (genetic marker). Several primers, including mitochondrial DNA cytochrome-b/Cyt-b (Indriati & Yuniarsih, 2019), 12S rRNA (Cahyadi et al., 2017), D-Loop (Karabasanavar et al., 2017), and NADH dehydrogenase-4 (ND4), have been employed successfully in detecting pork DNA fragments (Kusnadi et al., 2021). The primers utilised in this work were Cyt-b and ND4, which have previously successfully detected pork DNA from processed meat products (Kusnadi et al., 2020; Kusnadi et al., 2024). To our knowledge, there is no evidence that ND4 can identify pig DNA fragments, and no references compare ND4's performance to that of Cyt-b.

DNA isolation is typically performed before the PCR process to isolate pure DNA. According to Gargouri and Kacem (2018), a variety of DNA extraction protocols involving phenol-chloroform-isoamyl alcohol and others are effective but time-consuming and require many steps. Direct PCR is an alternative approach to combine the cell lysis step with DNA amplification in one reaction (Guan et al., 2019). The lysis buffer approach can be utilised to shorten the time required for DNA isolation while also being cost-effective. Using the lysis buffer will lyse the cell wall, allowing the components within the cell to escape. This is referred to as the direct-PCR approach (Gupta, 2019). Therefore, an effective detection method for identifying pork contaminants is urgently needed and critical for food safety (Ha et al., 2017).

A limited study has shown the capability of direct PCR detection of pork DNA using Cyt-b and ND4 primers. Furthermore, the optimisation of lysis buffer conditions in direct PCR applications has not been thoroughly investigated to improve detection consistency in commercial products. Hence, the current study used the direct PCR method and two primers to detect pork DNA to avoid adulteration of processed meat products while reducing the time and expense required.

MATERIALS AND METHODS

Time and Location

The present study was carried out at the Biology and Molecular Laboratory, Central Laboratory of Life Sciences (LSIH), Universitas Brawijaya, Malang, Indonesia. The study was carried out between May and September 2021.

Sample Collection and Preparation

Pork meat and processed meat items such as meatballs, sausages, and corned pork were used as samples in this study. This study's sample was obtained from street vendors, traditional markets, and modern marketplaces in Malang City. Each of the four samples was weighed (0.25 g), wrapped in aluminium foil, and labelled according to the sample type. Samples were then stored at -20°C for further analysis.

Sampling Test

Lysis buffer and incubation conditions were tested upon sampling by direct PCR. The temperature of 95°C for 5 min was selected as the optimal lysis temperature in all samples (three brands × three product types × three replicates). The DNA was then measured for concentration and quality before amplification with ND4 and Cyt-b primers and then visualised using electrophoresis.

Direct PCR (Cell Lysis)

The direct-PCR approach bypasses the need for DNA isolation, as does the normal PCR method, and instead employs a lysis buffer to obtain DNA from cells. Weighed meat and processed product samples were put in a 1.5 ml Eppendorf tube containing 200 µL of lysis buffer formulation: 0.2 M NaOH, 0.025 M EDTA, 0.1 M NaCl, 0.01 % SDS, 0.01 Tris-Cl, and 0.07 % Tween-20 based on the modified approach (Guan et al., 2019), and incubated (Memmert) at various temperature (85°C and 95°C) and incubation time (5, 10, and 15 minutes) to achieve optimum condition. Each sample was incubated at 800 rpm in a Thermomixer (HLC BioTech/MHR 13) with this treatment, and then 45 µL of Tween 20 was added. The samples were kept in a 4°C refrigerator (Toshiba) for 15 min before being tested for concentration and purity.

DNA Concentration and Purity Assessment

DNA concentration and purity were assessed with a nanodrop spectrophotometer (ND-1000 UV/Vis) at 260 nm and 280 nm. The purity of DNA was determined by the A260/A280 ratio, while the concentration of DNA was expressed in ng/µl.

PCR Amplification

PCR was performed using two sets of species-specific primers for the mitochondrial genes ND4 and Cyt-b. Each 10 µL PCR reaction contained 0.5 µL forward primer (5 pmol/µL), 0.5 µL reverse primer (5 pmol/ µL), 3 µL ddH_2O_3, 5 µL MyTaq™ Red Mix (Bioline), and 1 µL of DNA template. To avoid reagent breakdown, low temperature conditions were maintained. The tube from the PCR mix was gently mixed and spun down into 1.5 mL tubes. PCR reactions were performed in 0.2 mL thin-walled tubes with a thermal cycler (Applied Biosystem™ PCR System 9700 Thermal Cycler, Thermo Fisher Scientific, USA). PCR condition was set at initial denaturation at 94°C for 5 s, and then 32 cycles with denaturation (94°C for 30 s), annealing (55-60°C for 45 s), extension (72°C for 30 s), followed by final extension at 72°C for 5 min. The results were confirmed by electrophoresis (Guan et al., 2019).

Electrophoresis

PCR amplified results were subjected to horizontal electrophoresis with Mupid 2 Plus System (Takara Bio Inc., Japan). Then it was visualised on a 1.5% agarose gel. Agarose powder was dissolved in 1x Tris-Borate-EDTA (TBE) buffer. 2 µL of ethidium bromide was added to visualise the DNA, and then poured into an electrophoretic gel mould. A comb and electrophoretic plate were inserted into the mould, and the gel was allowed to solidify for 30-40 min at room temperature under closed conditions. After solidification, the gel was submerged in 1× TBE buffer. A total of 5 µL of PCR product (amplified DNA) mixed with 1 µL loading dye was loaded into each well, along with 3 µL of 100 bp DNA ladder as a size reference. Electrophoresis was performed at 50 V for 50-60 minutes. DNA bands were visualised and documented using a gel imaging system (Bio-Rad Chemidoc™/ BR-200, Bio-Rad Laboratories Inc., USA)

RESULTS AND DISCUSSION

Optimisation of Lysis Buffer Conditions

The optimisation of DNA extraction was carried out by varying lysis buffer incubation temperatures (85°C and 95°C) and durations (5, 10, and 15 minutes). The results showed DNA purity ranging from 1.70 to 2.25 and DNA concentrations between 66.17 and 469.23 ng/µL (Table 1).

DNA purity is assessed based on the A260/A280 ratio. Regarding Hashemipetroudi et al. (2018), there is typically no protein and RNA contamination when the values are between 1.80 and 2.00. Nucleic acids' absorbance at 260 nm and contamination with protein are at 280 nm (Setiati et al., 2020). The majority of results were outside of the allowed range with slight contamination, possibly from protein (ratio <1.80) or RNA (ratio >2.00), especially in the sausage, 15-minute pork meat, and meatball samples. Most samples exhibited acceptable DNA quality and quantity for PCR amplification, with a few samples exhibiting deviations. These results demonstrated that an efficient method for obtaining amplifiable DNA from processed meat products is direct lysis at 95°C for 5 min.

Table 1
Optimisation results of buffer lysis conditions

Samples	Incubation time	Temperature	DNA concentration (ng/µL)	DNA purity
Pork meat	5	85	124.27	1.81
		95	98.07	1.92
	10	85	230.78	1.73
		95	213.79	1.84
	15	85	118.07	1.70
		95	180.01	1.74
Meatball	5	85	121.94	1.94
		95	66.17	1.95
	10	85	102.62	1.81
		95	101.41	1.83
	15	85	85.95	1.73
		95	180.89	1.73
Sausages	5	85	164.23	2.25
		95	469.23	2.11
	10	85	417.02	2.14
		95	205.77	2.14
	15	85	192.15	2.19
		95	407.79	2.14
Corned beef	5	85	191.53	1.81
		95	205.16	1.86
	10	85	79.14	1.89
		95	215.81	1.81
	15	85	193.51	1.83
		95	242.39	1.85

PCR Amplification with ND4 and Cyt-b Primers

The results showed that using multiple temperature and incubation time conditions, successful amplification was observed with the ND4 (120 bp) and Cyt-b (398 bp) (Figure 1). Blanks and negative controls showed no amplification, confirming the absence of contamination.

At 5 minutes of incubation, pork DNA was successfully amplified at both 85°C (A1, A3, A4) and 95°C (B1, B2, B3, B4) using ND4 primers, except in sample A2 (85°C), which yielded no band. Cyt-b amplification at this time point was successful across all samples. At 10 minutes, ND4 amplification was successful in some samples (C1, C3, D3, D4), but failed in others (C2, C4, D1, D2). Cyt-b amplification was generally successful, except in samples C4 (85°C) and D4 (95°C). At 15 minutes, most samples were successfully amplified with ND4, except E2 (85°C), which showed no band. Cyt-b amplification at this time point failed in E2, E4 (85°C), and F4 (95°C).

Possible causes of amplification failure include DNA degradation from high-temperature processing (Yahya et al., 2017), residual contaminants such as proteins, carbohydrates, or polysaccharides that inhibit Taq polymerase activity (Muflihah et al., 2023; Rezadoost et al., 2016), suboptimal annealing temperature, leading to either non-specific binding (too low) or poor primer-template interaction (too high) (Wijaya et al., 2022), and primer-template mismatch, particularly in degraded DNA (Deekshit et al., 2019). Izadpanah et al. (2018) also observed various band thicknesses. The larger bands suggest that there were more DNA templates (Setiati et al., 2020). Variation in ND4 and Cyt-b primer pairs reflects differences in product size or amplification efficiency. For decayed or processed products, ND4 with a smaller amplicon performed better (Kusnadi et al., 2020) than Cyt-b, which becomes more susceptible to degradation (Matange et al., 2021).

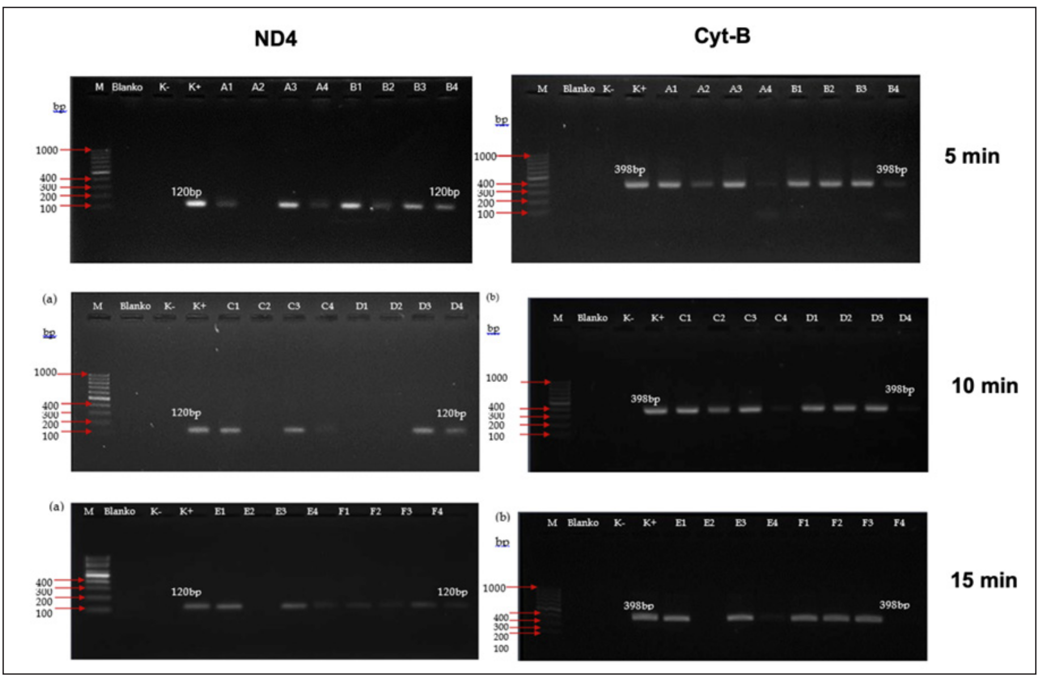

Figure 1. Visualisation of DNA bands on optimum buffer lysis conditions at incubation time of 5, 10, 15 min at 85°C and 95°C for both ND4 and Cyt-b. Note: 5 min treatment: K- = ddH2O, K+ = Pork meat isolated by Kit, A1 = Meat (5', 85°C), A2 = meatball (5', 85°C), A3 = sausage (5', 85°C), A4 = corned beef (5', 85°C), B1 = meat (5', 95°C), B2 = meatball (5', 95°C), B3 = sausage (5', 95°C), B4 = corned beef (5', 95°C); 10 min treatment: C1 = meat (10', 85°C), C2 = meatball (10', 85°C), C3 = sausage (10', 85°C), C4 = corned beef (10', 85°C), D1 = meat (10', 95°C), D2 = meatball (10', 95°C), D3 = sausage (10', 95°C), D4 = corned beef (10', 95°C); 15 min treatment: E1 = meat (15', 85°C), E2 = meatball (15', 85°C), E3 = sausage (15', 85°C), E4 = Corned beef (15', 85°C), F1 = meat (15', 95°C), F2 = meatball (15', 95°C), F3 = sausage (15', 95°C), F4 = corned beef (15', 95°C).

Parameters such as DNA extraction method, concentration, and purity of DNA, primers, and inhibitors such as proteins, phenols, RNA, and other substances in cells can affect DNA amplification success (Deekshit et al., 2019; Amiteye, 2021; Sophian et al., 2021). Furthermore, the polysaccharide and other organic content present could inhibit DNA polymerase enzymatic activities (Rezadoost et al., 2016). Primer specificity is crucial for food authenticity and detection of contaminants in small amounts (Arini et al., 2018). Differences in DNA intensity may occur due to DNA degradation throughout the food preparation process. DNA degradation happens when the links between molecules are disrupted due to extreme physical treatment, overheating, and the presence of certain chemicals (Bhoyar et al., 2024). This is consistent with prior research, which indicates that processed product samples are prone to DNA fragment degradation. The processed product has been blended with 38 other substances and heat-treated; therefore, there is a possibility that the DNA present in the sample has been degraded and its concentration has been reduced (Yahya et al., 2017).

Sampling Test Results

The sampling test used optimised conditions (lysis at 95°C for 5 min). DNA purity values ranged from 1.62 to 2.11, while concentrations varied between 126.09 and 321.43 ng/µL (Table 2). Some samples fell outside the 1.80-2.00 purity range, indicating minor contamination—likely protein contamination in meatballs and corned beef (A260/A280 <1.80), and RNA contamination in sausages (A260/A280 >2.00) (Hashemipetroudi et al., 2018).

Table 2
Concentration and purity of processed meat products

Samples	Concentration (mg/µL)	Purity (A260/A280)
Meatball A*	145.72	1.74
Meatball B	212.59	2.01
Meatball C	126.09	1.80
Sausage D*	321.43	2.11
Sausage E	192.54	1.95
Sausage F	274.63	2.08
Corned beef G*	168.82	1.86
Corned beef H	223.29	1.62
Corned beef I	168.43	1.62

Note. Asterisk (*) indicates good concentration and purity

Figure 2 shows that meatball brand A (A1-A3) was positive for pork DNA with both ND4 and Cyt-b primers, while brands B and C were negative. Sausage brand A also tested positive with both primers; brands B and C were negative. Because the ND4 and Cyt-b primers used in this study are specific for the pig species, it was indicated that commercial meatball brands B and C are not contaminated with pork DNA. For corned beef, all samples showed negative results with Cyt-b primers, while ND4 showed faint or inconsistent amplification. Corned beef brand A might be amplified more efficiently in Cyt-b than ND4 primers.

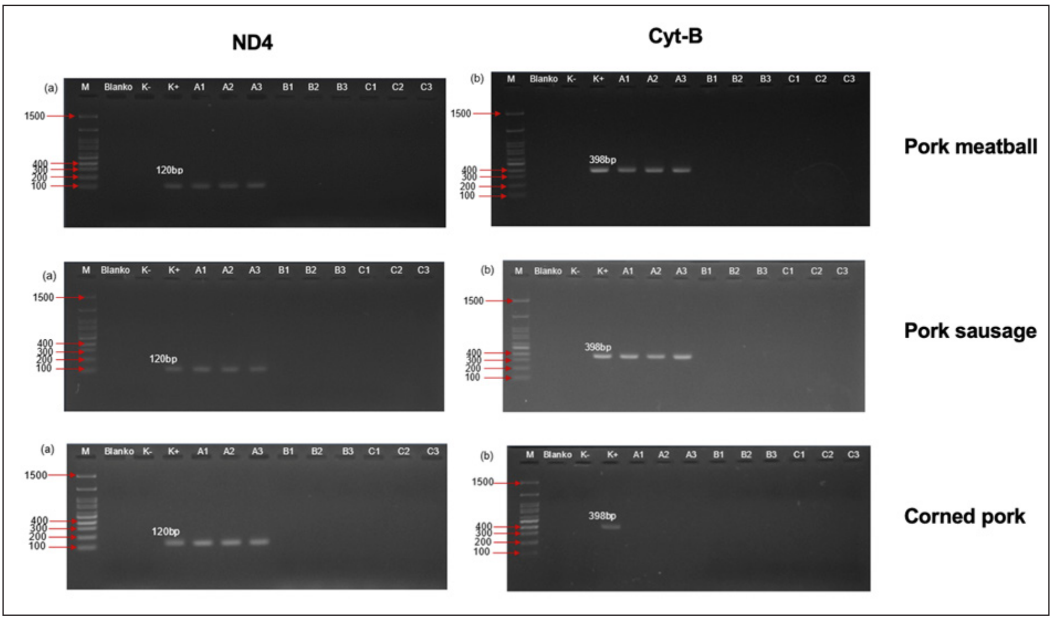

Figure 2. Visualisation of DNA bands on 3 meatballs, 3 sausages, and 3 corned beef brands with three repetitions for both ND4 and Cyt-b. Note: Meatball: K- = ddH_2O, K+ = Pork meat isolated by kit, A1-A3 = meatball brand A (contains pork), B1-B3 = meatball brand B (no pork), C1-C3 = meatball brand C (no pork); Sausage: A1-A3 = sausage brand A (contains pork), B1-B3 = sausage brand B (no pork), C1-C3 = sausage brand C (no pork); Corned beef: A1-A3 = corned beef brand A (contains pork), B1-B3 = corned beef brand B (no pork), C = corned pork brand C (no pork).

The absence of detectable DNA bands in some processed products may result from high-temperature sterilisation (120°C for 15 min), which compromises DNA stability (Matange et al., 2021), ineffective primer binding due to degraded DNA or excessive product length, especially for the longer Cyt-b amplicon (398 bp), and the presence of PCR inhibitors (Yahya et al., 2017). Corned pork is also sterilised at 120°C for 15 min. This treatment can have an impact on the stability of the DNA to be amplified since, while DNA is still present in the sample even when heated to 120°C, the stability of DNA has reduced, resulting in DNA degradation (Matange et al., 2021). Since Cyt-b is longer than the ND4 primer, PCR performance may be affected. According to Kusnadi (2020), overly long primers will make the amplification process less successful, resulting in the degradation of the amplified DNA band. The study findings revealed that the most effective method for detecting pork DNA in processed meat products is direct PCR using ND4 and Cyt-b primers with optimum lysis conditions established at 95°C for 5 min. When amplifying degraded DNA, ND4 was more suited for the processed product. There was an insufficient sample size and unmeasured sensitivity and specificity of the results. Therefore, validation using real-time PCR is needed to improve the sensitivity and accuracy.

CONCLUSION

The direct PCR approach can amplify DNA from fresh meat and processed meatballs, sausages, and corned pork products, utilising ND4 and Cyt-b primers. Compared to ND4, the Cyt-b primer was more effective at detecting pork DNA in processed products. The optimal temperature and incubation time were established at 95°C for 5, 10, and 15 min (Cyt-b), and 95°C for 5 min (ND4). More study with real-time PCR with primers ND4 and Cyt-b is needed to improve results (sensitivity), raise the accuracy of the smallest detectable DNA concentration, and quantify the amount of contamination in processed products.

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