Novel primer targeting the mitochondrial NADH dehydrogenase subunit 4 (ND4) and NADH dehydrogenase subunit 5 (ND5) for detection of porcine (Sus scrofa) DNA fragments in food products for halal authentication

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Abstract. In this research, a pair primer targeting NADH dehydrogenase subunit 4 (ND4) gene of porcine (Sus scrofa) mt-DNA was designed and tested for its specificity. The ND4 primer was compared for its robustness against an established primer pair designed to amplify the NADH dehydrogenase subunit 5 (ND5) gene. The samples include non-halal meat animals (dog, porcine, and rat) and halal meat animals (chicken, cow, and horse). DNA from raw meat was isolated by modified Chloroform Isoamyl-Alcohol method and then was analyzed quantitatively with NanoDrop™ spectrophotometer followed by amplification using PCR technique. The amplification results proved that the two pairs of primers were specific, resulting in amplification of 120 bp DNA target for ND4 and 227 bp for ND5. It can be concluded that the two primers can differentiate between halal and non-halal animals. However, to determine the sensitivity of each primer, further research is needed.

1. Introduction

Adulteration is an activity of adding or mixing with other substances that are not supposed to be in food products that cause a decrease in food quality. Adulteration can occur for several reasons like financial gain, carelessness, and inadequate food ingredients [22]. Adulteration in meat products mainly occurs by mixing poor quality meat with good ones. Porcine is one example of meat commonly used in adulteration. There is a high potential when porcine meat is used as an adulterant in beef, lamb, and chicken because of the affordable porcine price [18]. In Indonesia, most adulteration cases exist on food ingredients based on beef. Adulteration using porcine and its derivatives in food products are contradicted with Islamic Law according to ‘Halal.

Halal is an Arabic word that means a product which can be consumed by Muslims [1]. Non-halal food is prohibited to be consumed by Muslims besides in emergency conditions. Derivatives of porcine
and products containing porcine are examples of food that are forbidden to be consumed in Islamic Law. Hence, reliable detection methods to detect adulteration and non-halal components in food products are needed.

DNA analysis technique such as Polymerase Chain Reaction (PCR) is commonly used for adulteration and halal authentication analysis in meat. Analysis based on DNA is more specific and sensitive because it could be applied in fresh, frozen, processed, and even in degradation products. DNA analysis in meat using PCR normally uses genetic markers such as nuclear DNA or mitochondrial DNA (mt-DNA) [2] [9]. mt-DNA has several advantages over nuclear DNA. This is due to the amount of mt-DNA being more than nuclear DNA [16]. Furthermore, mt-DNA has a single allele because it was inherited from the mother [14] [15]. This makes mt-DNA unique in lineage tracing compared to nuclear DNA which is inherited from both parents and can cause ambiguity due to the high diversity between individuals [23]. Thus, mt-DNA is regularly used for designing primer pairs in analysis based on DNA in meat using PCR techniques.

A primer is a short sequence of DNA that flanks a specific target sequence to be amplified. The selection of DNA target sequence and primer pair design will influence the success of the PCR technique. Designing a primer is an important process to get a specific PCR product and accurate detection result. Primer pair is needed to strengthen target DNA which is wanted to be amplified, so satisfactory results were obtained [3] [12]. This study designed a primer pair to amplify the NADH dehydrogenase subunit 4 (ND4) gene to detect the presence of porcine DNA. This specificity and accuracy of the ND4 primer were then compared to a primer pair designed in a previous study targeting the NADH dehydrogenase subunit 5 (ND5) primer [8].

2. Material and methods

2.1. Materials

Fresh meat samples of chicken, beef, and porcine were purchased from various markets or supermarkets located in Malang City. Fresh wild boar meats were purchased via E-commerce. The fresh dog and horse meat were obtained from special outlets that sell both processed food. Fresh rat meat was obtained from Malang City. Fresh wild boar meats were purchased via E-commerce.

DNA isolation materials are STE buffer (100 mM NaCl, 10 mM Tris-Cl pH 8, 1 mM EDTA pH 8), 10% SDS, 10 mg/mL proteinase-K, 24:1 chloroform-isooamyl-alcohol, 5 M NaCl, absolute ethanol, 70% ethanol, and TE buffer pH 7.6. Materials for DNA amplification using PCR methods include ddH2O, Go-Taq Green Master Mix (Promega, M7122), 10 mg/mL Bovine Serum Albumin (Bioworld, V17102000) and 5 pmol/μl of each porcine-specific primer. The primer pair specifications are shown in Table 1. DNA electrophoreses materials include 1.5 % agarose powder (Genaxon Bioscience, D00276), Ethidium Bromide (Promega, 0000392341), 1x Tris Borate EDTA buffer pH 8 (Promega, 0000446046), loading dye (Promega, 0000296909) and 100 bp DNA ladder (Jena Bioscience, M214).

2.2. Tools

The study used tools include Analytic scale (BSA224S, Sartorius), Autoclave (BS 315, Tomy Seiko Ltd), Centrifuge (Mikro 22R, Hettich), Chemical Gel Imaging (BR-200, Bio-Rad), Horizontal Electrophoresis (Mupid 2 plus), Incubator (Memmert), Microwave (NN-S215/MF), Microcentrifuge (Bio-Rad and ViPR2), Micropipette (Vitlab), Microtiter, Nanodrop spectrophotometer (NanDrop/ND-1000 UV/Vis), Refrigerator (Samsung), Thermal cycler (CR system 9700, Applied Biosystem), Thermal cycler (Version III model TP 600, Takara), Thermomixer (MHR 13, HLC BioTech), Vortex (BR-200, Bio-Rad), knife, 1.5 mL microcentrifuge tube (Eppendorf), 50 mL tube (BD Falcon), microtube rack, 0.2 mL PCR tube rack, stainless steel scissor, tweezer, spatula, micro pestle, white tip, yellow tip, blue tip, 0.2 mL PCR tube, 100 mL Erlenmeyer, glass graduated measuring cylinders, and microtiter.
2.3. **Primer design**
A pair of a porcine specific-primer was designed targeting the ND4 gene complete sequence in the mt-DNA of porcine obtained from National Center for Biotechnology Information (NCBI) website. The GenBank accession number KJ789952.1 was selected as a reference sequence for *Sus scrofa domesticus* (domestic porcine). To get the specific site, ND4 sequence of porcine compare with dog (*Canis lupus familiaris*, KJ522809.1), chicken (*Gallus gallus*, KJ778617.1), horse (*Equus caballus*, NC_001664.1), beef (*Bos indicus*, AF492350.1) and rat (*Rattus norvegicus*, NC_001665.2). ND4 sequences from several species were aligned using ClustalX and BioEdit software to find conserved regions across species and determine unique sequences to porcine. Characteristics of good primers were considered of their product amplification, GC content 40-60%, Melting Temperature (Tm) 50 -60 °C, Annealing Temperature (Ta) max 5 °C from Tm, base repetitions 4 times and primer length 20 – 30 bp.

2.4. **Primer specificity test**

2.4.1. **DNA isolation**
Isolation of DNA was carried out using Chloroform Isoamyl-alcohol Method with slight modification to omit the use of phenol [20]. Twenty milligrams of the sample was placed in 1.5 mL Eppendorf tubes containing 500 µl of STE buffer. The sample was mashed using a micro pestle before adding 40 µl of SDS 10% and 20 µl of 10 mg/mL proteinase K. Afterwards, homogenized using vortex for 20 sec followed by overnight incubation in a thermomixer at 55 °C, 800 rpm.

The next day, the samples were centrifuged at 12000 rpm for 10 min at 29°C, then 400 µl supernatant was transferred into a new 1.5 mL tube. An equal volume of chloroform: isoamyl-alcohol (24:1) and 40 µl of 5 M NaCl were added followed by gentle inversion until homogenous, then the mixtures were centrifuged at 12000 rpm for 5 min at 29°C. This step was repeated two times, but the second repetition was without the addition of 5 M NaCl. Then, 200 µl supernatant was transferred into a new 1.5 mL tube. The 800 µl of cold absolute ethanol and 40 µl of 5 M NaCl were added and gentle inversion until homogeneous before incubating at -20 °C for 2.5 h.

After incubation, the mixture was centrifuged at 12000 rpm for 10 min at 4 °C. The pellet was added with 500 µl of 70% ethanol, then was centrifuged again at 12000 rpm for 5 min at 4 °C. The pellet was warmed up at a thermomixer at 55 °C until ethanol evaporated. The pellet was dissolved in 50 µl of TE buffer pH 7.6. The purity and concentration of DNA isolation were analyzed by NanoDrop™ spectrophotometer (NanoDrop/ND-1000 UV/Vis). The DNA isolates were stored at -20°C.

2.4.2. **Polymerase chain reaction (PCR)**
Primer specificity test was done using conventional PCR. The DNA isolated from target species, porcine and wild boar, and non-target species, dog, chicken, beef, horse and rat meat were amplified using the designed ND4 primer pair with the primer ND5 was used as comparison. One µl of DNA sample was added into 9 µl PCR mixture containing of 2.75 µl ddH2O, 5 µl Go Taq Green Master Mix, 0.25 µl BSA, 0.5 µl of forward primer and 0.5 µl of reverse primer in 0.2 mL tube. This PCR mix must be homogenization using spin down. Thermal cycler was setting at 95 °C for 5 min for hot start, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 54 °C for both primers in 1 min were obtained from optimization PCR annealing temperature and extension at 72 °C for 30 s. The last stage was final extension at 72 °C for 5 min. PCR products were stored at -20 °C before use for further analysis [5]. The PCR products were visualized in 1.5 % agarose that dissolved in 1x TBE buffer and run at 50V for 45 – 50 min. The electrophoresis result was visualization using chemical-gel imaging [13].
3. Results and discussion

3.1. 2ND4-porcine specific primer design
A pair of forward and reverse primers was designed from the ND4 gene in porcine mt-DNA (Sus scrofa). The primer design was carried out by aligning the ND4 gene sequences from dog, chicken, horse, cattle, and rat. The alignment results obtained a specific sequence area along 120 bp based on the high base sequence variation between the ND4 gene in porcine and the ND4 gene in other animals. This gives the primer a high potential for binding specifically to the ND4 DNA target sequence in porcine. The primer will be polymerized, so it must be able to attach to a specific target and the sequence between the primer and the target DNA must be complementary. The target amplicon of 120 bp was chosen because the amplicon size <150 bp is more stable to be applied to DNA samples that have been degraded [17]. Moreover using a long region of DNA template as a primer, increasing the possibility of hybridization with another primer which makes the DNA template doesn’t amplify [21]. The characteristics of the designed forward and reverse ND4 primers and ND5 primers as a comparison can be seen in Table 1.

| Gene Type                        | Sequence (5’ - 3’)                  | Length (bp) | % GC | Tm (°C) | Ta (°C) | PCR Product (bp) | Reference |
|----------------------------------|-------------------------------------|-------------|------|---------|---------|-----------------|-----------|
| NADH dehydrogenase sub unit 4 (ND4) | Forward: CCCATCCCATCAATCTAATCGG | 21          | 47.6 | 62      | 54      | 120             | This study |
|                                  | Reverse: ATGTAGAGAGAGTAGAGGGGC     | 20          | 50   | 60      | 62      |                 |           |
| NADH dehydrogenase sub unit 5 (ND5) | Forward: CATTGCGCTCCTCACTCATTAACC | 23          | 47.8 | 68      | 54      | 227             | [8]       |
|                                  | Reverse: AAGAGAGAGAGTTC TACGGTCTGTAG | 26          | 46   | 76      |         |                 |           |

Primer pairs for the porcine ND4 gene were designed to fulfill the characteristics of a good primer design. The sequence length of forwarding and the reverse primer is 21 bp and 20 bp, respectively. Specific primer pairs are ideally designed to range from 18 to 22 bp in length. If the primer is too short, it can cause mispriming (primer does not attach to the sequence target) and decrease the primer specificity level. However, a longer primer could risk the formation of hairpin or secondary structures. Lastly, the ND4 primer pair has a GC content of 47.6% for the forward primer and 50% for the reverse primer. The recommended GC content for primers should be between 45-60% [4].

3.2. DNA isolation and quantitative measurement
Isolation of DNA samples was carried out using the chloroform isoamyl-alcohol method. This method has several advantages, namely the general method used in laboratory procedures, effective to extract DNA from a small amount of tissue, produce high-quality DNA, affordable, and suitable for a wide range of application [7]. The DNA isolates obtained were identified quantitatively with a NanoDrop™ spectrophotometer. The results of quantitative measurements of sample DNA isolates are shown in Table 2.
assistance in the detection of halal meat products porcine and wild boar DNA will add to the collection of primers for halal detection and provide great.

nd5 cattle the lack of ability of the primers to amplify the studies, which resulted addition, specifically amplify porcine and wild boar DNA indicated by the single DNA band in lane 1 and 2. The DNA from horse, reaction to amplify the target sequence using DNA from porcine, compared for its specificity to the established specific primer only amplifies DNA segment from the target species. The novel of contamination. Each kind of meat has a specific characteristic and ingredients.

3.3. Porcine primer specificity test
The DNA from the target species, porcine, is compared with non-target species and showed that the specific primer only amplifies DNA segment from the target species. The novel ND4 primer was compared for its specificity to the established ND5 primer [8]. Here, the primers were used in a PCR reaction to amplify the target sequence using DNA from porcine, wild boar, chicken, cattle, dog, horse, rat as templates. The ddH2O was used as a negative control. The amplified DNA fragments were visualized on 1.5% agarose gel Figure 1.

The results show that the ND4 Figure 1a and ND5 Figure 1b primer pairs were only able to specifically amplify porcine and wild boar DNA indicated by the single DNA band in lane 1 and 2. In addition, the PCR product size is matched with the expected product size, i.e. 120 bp for the ND4 primer and 227bp for the ND5 primer. The specificity of ND5 primer is also in accordance with the previous studies, which resulted in a 227 bp DNA band when using DNA from porcine as a PCR template [8]. The lack of ability of the primers to amplify the non-target species i.e., horses, donkeys, lambs, and cattle, confirms its specificity. Both primers were designed based on the coding sequence of ND4 and ND5 genes which have different nucleotide sequences. Confirmation of the ability of ND4 to detect porcine and wild boar DNA will add to the collection of primers for halal detection and provide great assistance in the detection of halal meat products.

Table 2. Purity and concentration result of DNA samples.

| Raw meat samples | Concentration (ng/µl) | Purity (A260/A280) |
|-----------------|-----------------------|--------------------|
| Porcine         | 94.92                 | 1.99               |
| Wild boar       | 223.55                | 2.04               |
| Chicken         | 90.20                 | 1.72               |
| Beef            | 135.09                | 1.88               |
| Dog             | 50.31                 | 1.84               |
| Horse           | 77.33                 | 1.72               |
| Rat             | 143.37                | 2.01               |

The purity of DNA extracted from raw meat samples ranged from 1.72 to 2.04. DNA is considered pure when the absorbance value of A260/A280 ranges from 1.8 to 2.0. If the value is below 1.6, DNA is contaminated with protein, phenol, while values above 2.0 are RNA contamination [11]. DNA samples of chicken and horse meat still contained protein contamination. This contamination was thought to be due to the absence of phenol addition in the lysis buffer formulation. Phenol functions to bind proteins so that it can reduce protein residues in DNA isolates. However, the DNA extracted from raw meats was still in the range of 1.8 - 2.0 and can be concluded that the quality of isolated DNA was sufficient for PCR analysis.

The quantity of DNA isolated was depended on the kind of source of tissues. Fatty tissues had less amount of DNA rather than in kidney, liver, heart and tendon tissues [10]. The amounts of target DNA copies were varied in the different animals [6]. Also, there is a variation of protein in each meat due to variability in manufacturers’ process and production styles [19]. This variability also affects the variety of contamination. Each kind of meat has a specific characteristic and ingredients.
Figure 1. Visualization of Primer Specificity Performance of the mitochondrial (a) ND4 and (b) ND5-based porcine-specific PCR assay under raw meat. Lane M: 100 bp DNA ladder; Lane N: ddH2O, Lane 1: porcine; Lane 2: wild boar; Lane 3: chicken; Lane 4: beef; Lane 5: dog; Lane 6: horse, and Lane 7: rat.

4. Conclusions

Species-specific primers designed based on the ND4 gene were confirmed to be capable of detecting the DNA porcine and wild boar with similar effectivity with ND5 primers. Different from ND5 which produces a 227 bp amplification product, the ND4 primer produced a 120 bp amplification product of the target DNA. Therefore, both can be synergically used as molecular tools to detect and identify porcine genetic material. A comparison to the established ND5 primer pairs confirms the reliability of ND4 primer pairs to be applied in the detection of adulteration and halal authentication.

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