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SHORT COMMUNICATION

The employment of q-PCR using specific primer targeting on mitochondrial cytochrome-b gene for identification of wild boar meat in meatball samples

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ABSTRACT

Objective: The objective of this study was to employ real-time or quantitative polymerase chain reaction (q-PCR) using novel species specific primer (SSP) targeting on mitochondrial cytochrome-b of wild boar species (CYTBWB2-wb) gene for the identification of non-halal meat of wild boar meat (WBM) in meatball products.
Materials and Methods: The novel SSP of CYTBWB2-wb was designed by our group using PRIMERQUEST and NCBI software. DNA was extracted using propanol-chloroform-isoamyl alcohol method. The designed SSP was further subjected for validation protocols using DNA isolated from fresh meat and from meatball, which include specificity test, determination of efficiency, limit of detection and repeatability, and application of developed method for analysis of commercially meatball samples
Results: The results showed that CYTBWB2-wb was specific to wild boar species against other animal species with optimized annealing temperature of 59°C. The efficiency of q-PCR obtained was 91.9% which is acceptable according to the Codex Allimentarius Commission (2010). DNA, with as low as 5 pg/µl, could be detected using q-PCR with primer of CYTBWB2-wb. The developed method was also used for DNA analysis extracted from meatball samples commercially available.
Conclusion: q-PCR using CYTBWB2-wb primers targeting on mitochondrial cytochrome-b gene (forward: CGG TTC CCT CTT AGG CAT TT; Reverse: GGA TGA ACA GGC AGA TGA AGA) can be fruitfully used for the analysis of WBM in commercial meatball samples.

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CYTBWB2-wb primer; meatball; halal authentication; q-PCR; wild boar

Introduction

Meat is a good source for protein needed for human's cell development and is taken account as the best nutritional sources because of its taste. The awareness of community to consume protein-rich food has increased the consumption of meat recently. Some consumers are aware about meat which composed food products they eat; therefore, the accurate labeling is a must to give the consumer choice [1]. Some aspects must be considered regarding the consumption of meat, especially religion reasons in which Islamic and Jew followers are prohibited to consume certain meat types, such as pork and wild boar meat (WBM). Besides, meat consumption has been also correlated with certain diseases, such as mad-cow disease or bovine spongiform encephalopathy due to the consumption of beef [2]. Based on religion and health aspects, the identification of meat types for authenticity of meat-based foods is

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challenging and very important not only for consumers but also for regulators and producers [3].

In Asiatic region, meatball, known as *bakso* in Indonesia and *bebola* in Malaysia, is considered as favorite meat-based foods and is mainly composed of 90% meat. The most commonly meat used in meatball is beef and chicken [4]. But, due to the different price between beef as representative halal meat and non-halal meat, such as pork and WBM, meatball producers have tried to blend or substitute beef with pork and WBM [5]. The presence of WBM in any food products must be identified due to its status as non-halal meat in Muslim societies, as consequence, numerous analytical methods have been developed, standardized and used for identification and confirmation of WBM for the sake of halal authentication [6].

Instrumental analytical methods equipped with computer software have been reported for the authentication of halal and non-halal meats based on physico-chemical properties like infrared spectroscopy Fourier transformed in combination with several statistical multivariate analysis of classification and quantification [7,8], chromatographic-based, such as gas chromatography-mass spectrometry [9], liquid chromatography-mass spectrometry [10], immune-chromatography [11], differential scanning calorimetry [12], and biological based-markers, especially polymerase chain reaction either conventional or real-time for DNA analysis [13] and a lateral flow device of immune-chromatographic systems [14].

Polymerase chain reaction (PCR) has emerged as the powerful analytical tools to identify meat species present in meat-based foods like meatball through deoxy-ribonucleic acid (DNA) analysis [15]. DNAs have been a target of analysis because they contain all the genetic information in organism. It is impossible to analyze all DNA molecules; therefore, only specific genes to certain species are evaluated using PCR [16]. PCR targeting on specific genes have been reported for analysis of WBM, including cytochrome-b (CytbAG3A) [17], mitochondrial (mt-12S rRNA) [18], and d-loop mitochondrial gene [19,20].

The detection of WBM using multilevel PCR and PCR combined with restriction fragment length polymorphism enzyme has also carried out by Parkanyi et al. [20] and Mutalib et al. [18]. However, these methods could quantify the total DNAs. Besides, these methods also need additional process that is electrophoresis to check the amplification products. These shortcomings can be overcome using real time PCR where amplification reactions can be observed directly with a high level of sensitivity, simpler, and faster process [21]. Arini et al. [19] have developed new primers for WBM detection; however, the designed primers could not able to distinguish between WBM and pork because both DNAs are equally amplified. CytbAG3A primers could specifically amplified DNA from WBM, but limit of detection (LOD) value obtained is still quite high, i.e., 48 pg/µl. Therefore, a new primer must be designed capable of detecting trace DNA in WBM for the sake of halal authentication.

In this study, real-time PCR using new species specific primer (SSP) targeting on mitochondrial gene using primer of CYTBWB2-wb was developed and validated for analysis of WBM in meatball intended for halal authentication analysis. The developed primary has low LoD value of 5 pg/µl which is suitable for the analysis of DNA contamination.

Materials and Methods

Samples

Wild boar meat, WBM, was obtained from Palangkaraya, Central of Kalimantan, Indonesia. Beef, pork, goat, chicken, rabbit, and dog meat were purchased from several slaughter houses around Yogyakarta, Indonesia. Laboratory prepared meatball were prepared by emulsifying 90% meats with different concentration and 10% of spices and other components as in Rohman et al. [4]. The commercial meatball samples were purchased from different local markets in around Yogyakarta, Indonesia.

Primers design

Forward (F) and Reversed (R) primers which are specific primer to DNA of wild boar were designed using Primerquest software from Integrated DNA Technologies. GenBank from NCBI was used to retrieve DNA sequences of mitochondrial cytochrome-b (Accession No. NC_026992). The primer specificity was checked silico using BLAST (primer-basic local alignment search tool) allowing the identification of local regions of homolog sequences which are similar to different animal species. The designed F and R primers used were:

Forward: CGG TTC CCT CTT AGG CAT TT

Reverse: GGA TGA ACA GGC AGA TGA AGA

The primers have the characteristics of melting temperature of 60oC (F and R), GC contents of 50% (F) and 47.6% (R) with amplicon product of 191 base pair (bp).

The extraction of DNA

DNA was extracted according to Maryam et al. [22] with slight modification consisting three steps, namely, lysis, extraction, and separation. Briefly, 200 mg of meat was blended in the mortar and subjected to lysis procedure in 2-ml micro-tube. The blended meats were added with 700 µl buffer lysis (consisting of Tris HCl pH 8.0, EDTA pH 8.0, Na-acetate pH 5.2, NaCl, and SDS 1%), 30 µl proteinase K (20 mg/ml), and homogenized using vortex for 5 min. This mixture was incubated at 65°C for 2 h in water bath (IK HB 10, Medford). The mixture was added with

one volume of phenol-chloroform-isoamyl alcohol and shaken occasionally using shaker for 30 min. The sample was centrifuged at 12,000 for 5 min using micro-centrifuge (Sartorius 3-30K Sigma, Germany). The supernatant was taken and added with 1× cold 2-propanol (Merck, Darmstadt, Germany). The mixture was then incubated in freezer for 15 h and then subjected to centrifugation at 12,000 rpm for 5 min. The supernatant was taken, added with chloroform (1:1 v/v), and subjected to centrifugation at 13,000 × g for 10 min. DNA was precipitated and supernatant was discharged. Ethanol 70% (500 µl) was added into micro-tube and the mixture was centrifuged 12,000 rpm for 5 min. The supernatant was removed and the residue was dried until DNA pellet was formed. DNA was then dissolved in 100 µl TE buffer, homogenized and incubated at 50°C. DNA isolated was then used for q-PCR analysis.

Evaluation of DNA

DNAs extracted from fresh meat and meatballs (prepared in the laboratory and commercial samples) were visualized with electrophoresis procedure (i-Mupid J Cosmo Bio Co., Tokyo, Japan) as in Rahmawati et al. [23] using agarose gel 0.8% in 1× TBE buffer comprising of tris base, boric acid, and EDTA 0.5M (pH 8) for 60 min at 90 V. The agarose gel was stained using GelRedTM from Biotium (Fremont, CA). DNA separated was visualized using UV light. The image of electrophoresis results was digitally recorded with transluminator (Syngene, Synoptics Ltd., England). The purity of DNA was quantitatively analyzed with Spectrophotometer NanoVueTM Plus from GE Healthcare (Buckinghamshire, UK). The levels of DNAs extracted were monitored by measuring absorbance values at wavelength of 260 nm, in which 1 AU (absorbance unit) is corresponding to 50 ng/µl of DNA, while DNA purity was calculated based on ratio (*R*) of absorbance values at λ 260 and 280 nm. DNA having *R* values of 1.7–2.0 were considered as pure [19].

Amplification analysis of DNA using q-PCR

The extracted DNAs were subjected to q-PCR according to the manufacturer's procedure given (Biotium Inc., Bio-Rad laboratories, CA). In reaction tube, 10 µl of Eva Green, 7 µl nuclease-free water, 1 µl forward primer 10.0 µM, 1 µl reverse primer 10 µM, and 1 µl of extracted DNA 50 ng were mixed and analysed using q-PCR instrument (CFX96 Touch Real-Time PCR Detection System, Biorad USA). The following procedure and temperature were followed: pre-denaturation at 95°C for 3 min (1 cycle), denaturation at 95°C for 10 sec (30 cycles), annealing at optimized temperature of 59°C for 20 sec, and extension or amplification at 72°C for 45 sec. The relative fluorescence signal was performed automatically at each cycle end. For analysis of melting curve, the temperature was set 65°C–95°C with increasing temperature of 0.5°C/5 sec. Data were processed using of CFX Maestro™ software included in q-PCR instrument.

Validation and application of q-PCR

q-PCR using designed SSP was subjected to validation procedure according to Codex Allimentarius Commission [24] by determining several characteristics performances, including amplification efficiency, LOD, and repeatability. The validated q- PCR method was employed for DNA analysis of meat species in commercial meatballs samples.

Results and Discussion

Because of high specificity and sensitivity, quantitative polymerase chain reaction (q-PCR) has emerged as method of choice for DNA analysis in non-halal meats, such as pork and WBM, intended to halal authentication. PCR can be considered as simple technique to use and to provide reliable results. Besides, PCR-based methods are potential as a standard method for DNA analysis due to its nature as fingerprint analytical technique. In this study, q-PCR using SSP was developed for analysis of WBM (non-halal meat) in meatball products. The first step is designing SSP targeting on mitochondrial cytochrome-b (CYTBWB2-wb). The selection of mitochondrial DNA because most cells contain multiple copies of mitochondrial DNA, high mutation rate, and offers low detection limit [11]. In addition, mitochondrial DNA also does not have protective proteins so that it is easier to isolate and to purify for further analysis [25].

Primer CYTBWB2-wb was used to amplify DNAs extracted from WBM and meatballs containing WBM. Extraction of DNA was performed in three steps, namely, lysis, extraction, and precipitation based on phenol-chloroform-isoamyl alcohol method. The quality of DNAs extracted was observed using electrophoresis on gel agarose 0.8% and visualized using UV light. The results (Fig. 1) showed that DNAs extracted from fresh meats, including pork, wild boar, beef, canine, chicken, rabbit, goat, and DNA extracted from laboratory-prepared meatballs have not degraded during the excessive extraction. The extracted DNA was then checked for its purity by measuring the absorbance values of solution containing DNA at 280 and 260 nm. The purity index of DNAs extracted along with its concentration was compiled in Table 1. DNAs having *R* values of 1.7–2.0 were considered as pure [19]. The high purity of DNAs extracted indicated that processing with heat as in meatball preparation do not cause DNAs to degrade.

Optimization of q-PCR condition

Four primers intended to mitochondrial cytochrome-b gene species have been designed, namely, two primers for pork analysis (CYTBWB2-p and CYTBWB4-p) and

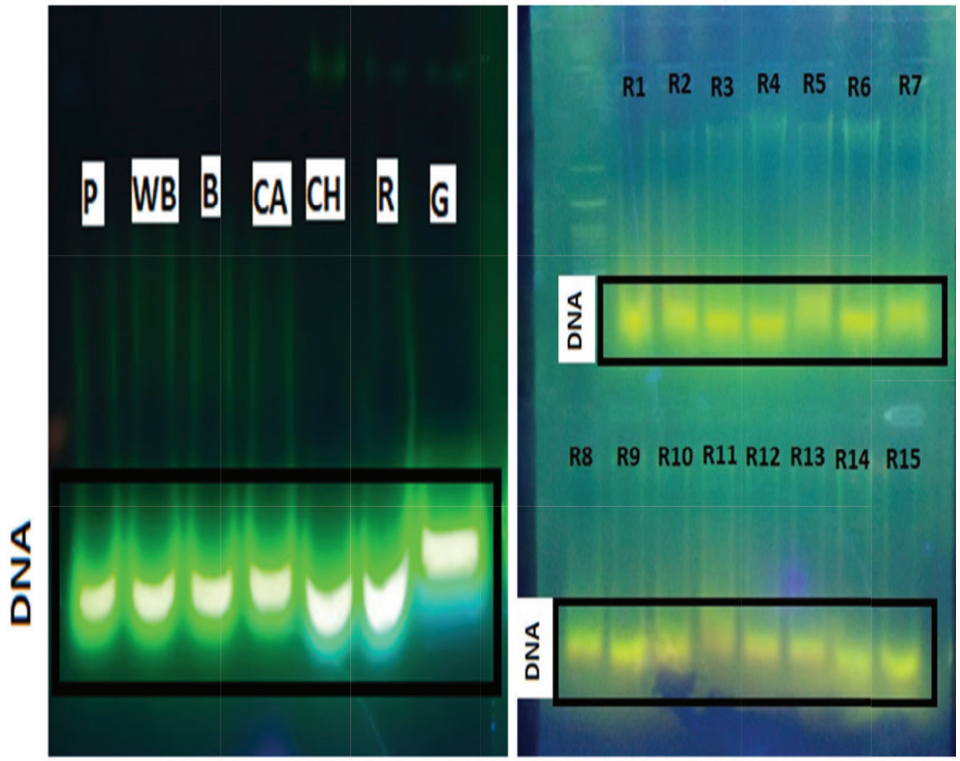

Figure 1. The evaluation of DNAs extracted using agarose gel electrophoresis from fresh meat of pork (P), wild boar (WB), beef (B), canine (CA), chicken (CH), rabbit (R), and goat (G) on the agarose gel 0.8%; [B] agarose gel electrophoresis of DNA extracted from reference meatballs containing wild boar at concentrations of 0,1% (R2), 0,3% (R3), 0,5% (R4), 0,7% (R5), 0,9% (R6), 1% (R7), 2% (R8), 3% (R9), 5% (R10), 10% (R11), 25% (R12), 50% (R13), 75% (R14), 100% (R15), as well as meatball containing beef 100% (R1)

Table 1. The purity index (ratio absorbance values at 260/280 nm) of DNAs extracted from several fresh meats.

Meats	260 (nm)	280 (nm)	Concentration (ng/µl)	Ratio 260/280
Pork	1.9527	0.9746	1,952.68	2.00
Wild boar	3.1502	1.5661	3,150.23	1.87
Beef	0.1060	0.0707	105.98	1.70
Canine	2.7870	1.5295	2,787.02	1.82
Chicken	3.6008	2.0049	3,600.84	1.80
Rabbit	0.1552	0.0885	155.21	1.75
Goat	0.2751	0.1526	275.13	1.80

two primers for wild boar analysis (CYTBWB2-wb and CYTBWB4-wb). Pork and wild boar have high homology so that both DNAs from pork and WBM could be amplified simultaneously. Figure 2 revealed the amplification curve along melting curve analysis (MCA) of primer CYTBWB2-p and CYTBWB4-p (for pork DNA) analysis and primer of CYTBWB2-wb and CYTBWB4-wb (for wild boar DNA). All the primers could amplify DNA templates accurately.

Among these primers, CYTBWB2-wb primer was selected for next optimization in terms of annealing temperature, an important factor in the process of q-PCR amplification which is associated with a primary attachment of primers on a target DNA. The initial annealing temperature used was based on around theoretical melting temperature (60°C), namely, at 58°C, 59°C, 60.2°C, and 61.2°C using 35 cycles. The temperature variation did not affect the shifting of quantification cycle (Cq) and response

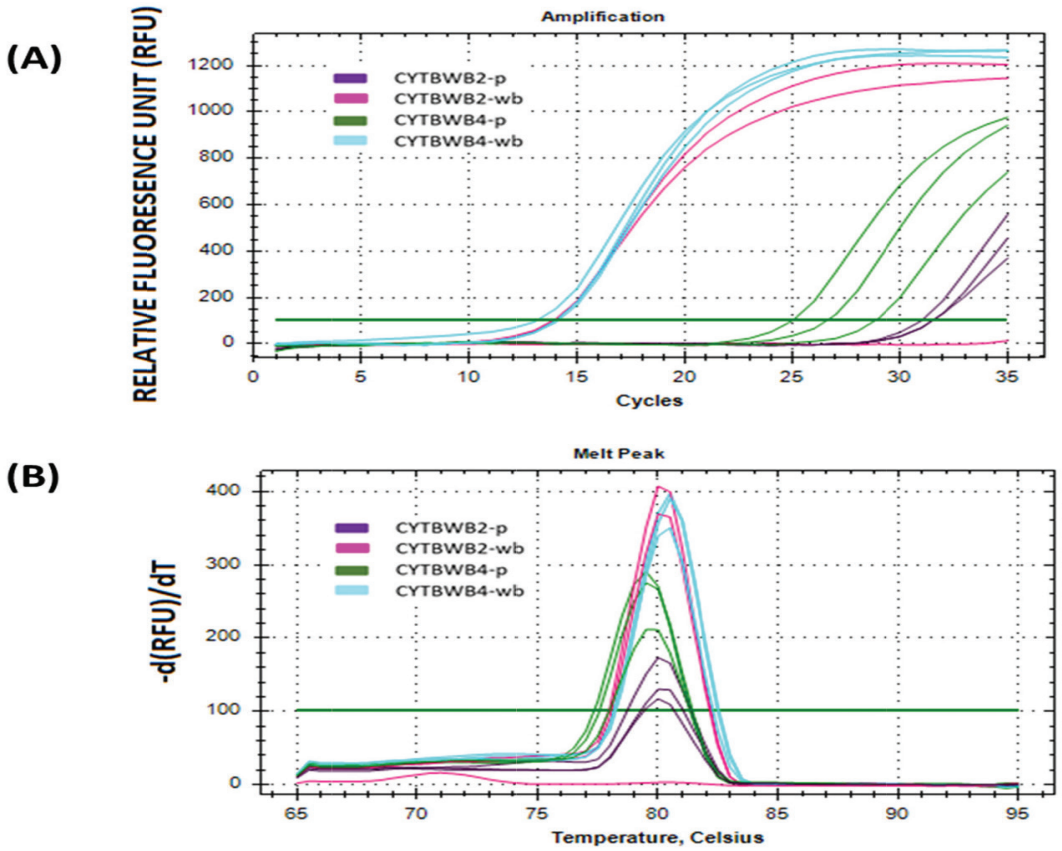

Figure 2. The amplification curve (A) along melting curve analysis (B) of primer CYTBWB2-p and CYTBWB4-p (for pork DNA) and primer of CYTBWB2-wb and CYTBWB4-wb (for wild boar DNA)

of relative fluorescence unit significantly in wild boar but was significant in pork DNA. The optimization results indicated that CYTBWB2-wb primer had large cycle range of amplification between pork and wild boar compared to primer CYTBWB4-wb, i.e., more than 30 cycles. The difference in amplification cycle is advantageous for analysis of WBM so that analyst can cut the amplification cycles allowing analysis of pork and WBM simultaneously. From optimization, the annealing temperature of 59°C was selected due to its capability to provide high response on wild boar DNA (369.22) and low response on pork DNA (130.39) along with large difference in amplification cycles, namely, 14.78 for wild boar and 32.37 for pork. For further analysis, 30 amplification cycles were applied during validation and analysis of commercial meatball samples.

Validation of q-PCR for analysis of wild boar DNA

Some performances characteristics, namely, primer specificity, amplification efficiency, LOD, and repeatability were evaluated during validation of q-PCR for analysis of wild boar DNA. Primer of CYTBWB2-wb was subjected to specificity test against DNA templates isolated from pork, wild boar, beef, canine, chicken, rabbit, and goat. Figure 3 revealed the amplification curve of CYTBWB2-wb indicating that primer could specifically amplify DNA from WBM and did not amplify DNAs from other meats and NTC (no template control).

The sensitivity evaluation of q-PCR system using primer of CYTBWB2-wb was determined by calculating LOD, the lowest DNA could be detected even not reliably quantified using q-PCR system. The value of LoD was determined by amplifying DNAs extracted from fresh WBM, serially diluted at different concentrations, namely, 10,000, 5,000, 1,000, 500, 100, 50, 10, 5, 1, and 0.5 pg/µl. The LoD of WBM DNA found was 5 ng/µl, as indicated that at this concentration DNA could be amplified but DNA at lower 5 ng/µl could not be amplified (Fig. 4). For determination of amplification efficiency (E), standard curves were constructed by correlating initial log DNA at concentration levels of 50,000, 10,000, 5,000, 1,000, 50, 10, 5, 1, and 0.5 pg/µL (x-axis) and Ct (Cq, quantification cycle) (y-axis), as shown in Figure 5. The coefficient of determination (R^2) value of

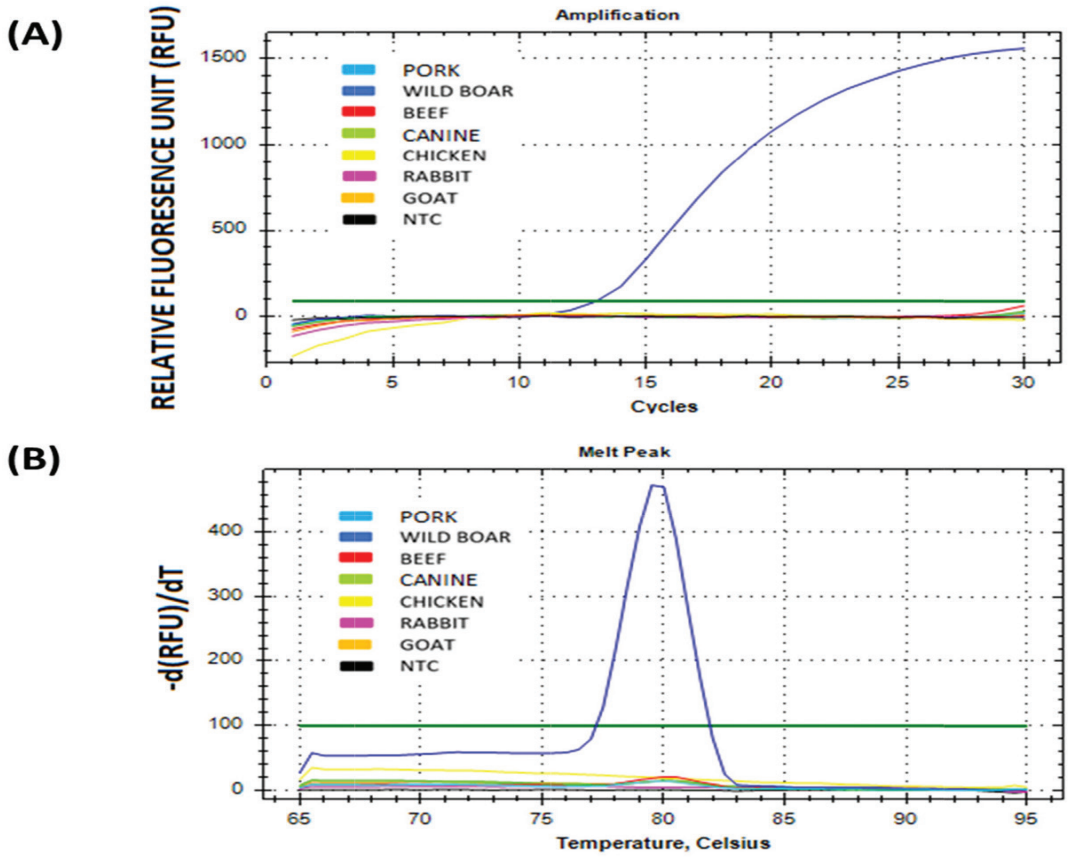

Figure 3. The amplification curve (A) along melting curve analysis (B) of primer CYTBWB2-wb using different DNA templates from animal species. Primer CYTBWB2-wb only amplified DNA from wild boar

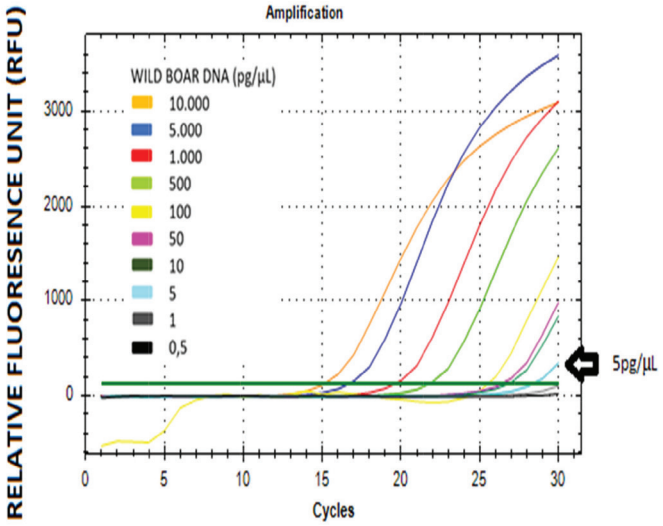

Figure 4. The sensitivity evaluation of primer CYTBWB2-wb for determination of LoD value of wild boar DNA

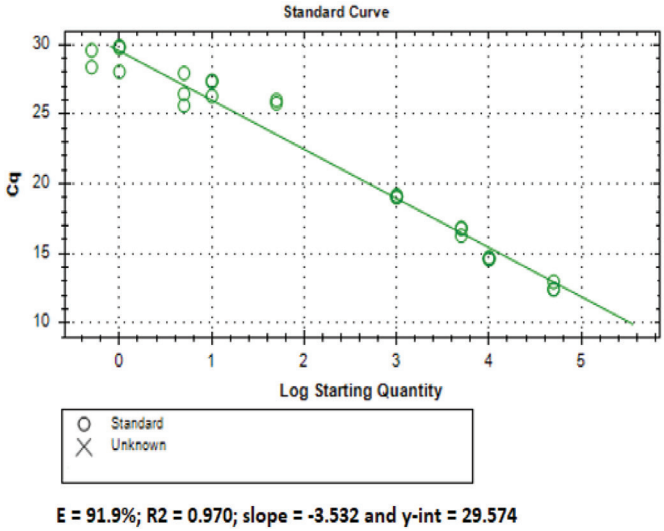

Figure 5. Standard curve describing the linear relationship between log10 of DNA concentrations extracted from wild boar meat and Cq (quantification cycles) values for determination of LOD and efficiency of amplification (E)

linear regression of 0.970, slope = −3.532, and *y*-intercept of 29.547 with *E*-value of 91.9%. These values obtained meet criteria of linearity curve according to standard values as in Widyasari et al. [26].

The precision of q-PCR for analysis of wild boar DNA was determined by repeatability test by replication of amplification of DNA at fixed concentration at 50 ng five times, and the relative standard deviation (RSD) of Cq values was determined. RSD value obtained was 20.98%, lower than maximum RSD value acceptable for q-PCR quantitative assay according to Codex Allimentarius Commission [24]. Based on these parameters, it could be concluded that q-PCR using CYTBWB2-wb was valid to be used as the analytical method for identification of meat species in commercial meatball samples. There were no amplifications observed during analysis of DNAs extracted from commercial meatball samples indicating that all the evaluated samples did not contain WBM in its products.

Conclusion

Primer CYTBWB2-wb was specific to wild boar species with detection limit of as low as 5 pg/µl. The validated q-PCR method using primer CYTBWB2-wb could be employed for analysis of DNA in commercial meatball samples. The developed method could be proposed as official method for halal authentication analysis of WBM in meatball products, supporting Indonesian Act on Halal Assurance systems (UU No. 33 year 2014).

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Conflict of interest

The authors have declared "no conflicts of interest with respect to the research, authorship, and/or publication of this paper".

Authors' contribution

GQA, YE, and AR performed research activity, compiled data, and prepared manuscript. MH, MRJ, MEA, and AR prepared manuscript and made critical thinking on the manuscript.

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