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Full Length Article

Qualitative and Quantitative Analysis of Canine (*Canis lupus familiaris*) Meat in Meatballs for Halal Authentication Study using Real-time Polymerase Chain Reaction

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Abstract

Canine meat (CM), one of non-halal meats having low price value, may be used as substitute or meat adulterant in beef meatballs by unethical producers to get economic benefits. This study was intended to design and to validate specific primer targeting on cytochrome-b gene for detection of CM in meatballs using real-time polymerase chain reaction (real-time PCR). The primer was *in silico* designed and was subjected to specificity test against DNAs extracted from several meats. The evaluation of other validation parameters for quantitative analysis was also performed including linearity, efficiency, sensitivity, repeatability and its application to commercial samples. The designed primer (CYTBCA3-kh) consisted of *F:* CCT TAG CCA ATG CCT ATT C and *R:* GCG ACT TGT CCG ATA ATG. The results showed that CYTBCA3-kh primer was specific to DNA extracted from CM and that extracted from meatballs containing CM using optimized annealing temperature of 50.6°C. The detection limit reported was 50 pg DNA corresponding to 0.1% CM in meatballs containing CM and beef. The relative standard deviation (RSD) for precision assay met the required acceptance criteria. The validated real-time PCR using CYTBCA3-kh primer could successfully identify CM in meatball formulation for halal authentication analysis. The developed method was potential to be developed as official standard method for CM detection in meatball products. © 2020 Friends Science Publishers

Keywords: Canine meat; Real-time PCR; Species-specific primer; Halal authentication; Meatballs

Introduction

Currently, there is an awareness increase among food consumers related to the safety issues and halalness status of food products, especially those from animal origin such as sausages and meatballs (Rodríguez-Ramírez *et al*. 2011). This phenomenon has attracted food scientist to provide reliable analytical methods for the authentication purposes, therefore any adulteration practices could be easily detected. Due to the discrepancy in price in major Muslim countries like Indonesia and Malaysia, some unethical meat sellers substitute beef with non-halal meats like canine meat, pork, and wild boar meat to get economic benefits (Nakyinsige *et al*. 2012). The addition of non-halal meats intentionally through adulteration practice or unintentionally action due to contamination is problematic toward Muslim communities because they are prohibited to eat these meats (Ali *et al*. 2013). Besides, these meats also act as carrier of zoonotic diseases, therefore, they are not hygienic and unsafe for consumption. This attracted some food scientist to develop meat's detection in food products.

Numerous analytical methods either qualitatively or quantitatively have been developed, validated and proposed to be used for analysis of non-halal meat including pork, wild boar meat, and canine meat (CM) in the pure and in food products such as vibrational spectroscopies (Guntarti and Abidin 2018; Rahayu *et al*. 2018a), gas chromatography in combination with mass spectrometry (Guntarti 2018; Rahayu *et al*. 2018b) and DNA-based methods such as polymerase chain reaction (PCR). Analysis through DNA offered specific means for analysis of meat types due to its property as fingerprint meat detection. DNA-based methods provided the potential application in halal authentication analysis of meat-based foods because DNA is considered as stable and not tissue dependent (Rodríguez-Ramírez *et al*. 2011).

Real-time PCR as reliable analytical method for DNA detection in meats using specific primer has been widely

reported for the identification and confirmation of non-halal meats. Specific primers targeting on mitochondrial cytochrome-b gene has been reported for detection of pork (Orbayinyah *et al*. 2019), canine meat (Rahman *et al*. 2014), and wild boar meat (Arini *et al*. 2018) in food products. Besides, the development of multiplex PCR assay (Prusakova *et al*. 2018) such as two direct-triplex real-time PCR with low resolution melting (Thanakiatkrai and Kitpipit 2017) and pentaplex PCR assay (Thanakiatkrai *et al*. 2019) has also used for the identification of non-halal meats. The present study aimed to design specific primer coupled with real-time PCR used for detection of canine meat in meatball products for halal authentication analysis.

Materials and Methods

Experimental details and treatments

Experimental materials: Dog meat was obtained from district Bantul, Yogyakarta Province, Indonesia. The others were obtained from Yogyakarta, Indonesia, in which goat, beef, and chicken were purchased from sub-district Mlati, Sleman, Yogyakarta, pork was bought from Godean, Sleman, Yogyakarta, rabbit was came from Kaliurang, Sleman, Yogyakarta, and wild boar meat was obtained from hunters in sub-district Baamang, district Kotawaringin Timur, Central Kalimantan Province, Indonesia.

Primers design: The primer design (CYTBCA3-kh) was performed using PrimerQuest software from Integrated DNA Technologies. During primer designing, the DNA sequence of canine mitochondrion complete genome (KM_113774.1) was retrieved from NCBI GenBank. The primer specificity was checked *in silico* using primer BLAST compared with beef, pork, wildboar, goat, chicken, rabbit, and rat. The designed primers used were:

Forward: CCT TAG CCA ATG CCT ATT C
Reverse: GCG ACT TGT CCG ATA ATG

The designed primers exhibited the characteristics of melting temperature of 53.3°C (F and R), GC contents of 47.37% (F) and 50.00% (R) with amplicon product of 105 base pair (bp).

The extraction and evaluation of DNA: The DNAs used for PCR assay were extracted manually using Phenol-chloroform, isoamyl alcohol (CIAA) methods. The purity of DNA was quantitatively analyzed with NanoQuant Spark Tecan (Switzerland) and was visualized with electrophoresis procedure (i-Mupid J Cosmo Bio Co., Tokyo, Japan). DNA purity was calculated based on ratio (R) of absorbance values at wavelengths (λ) of 260 and 280 nm, respectively. DNAs with R values of 1.8–2.0 were considered as pure.

Amplification analysis of DNA using PCR: The extracted DNA was analyzed using PCR. The reagent composition (20 μL) consisted of 10 μL EvaGreen PCR master mix, 1 μL forward primer and reverse primer, 1 μL DNA template (50 ng / μL), and 7 μL free nuclease water. The conditions used were: pre-denaturation at 95°C for 3 min (1 cycle), denaturation at 95°C for 15 sec, annealing at optimized temperature of 50,6°C for 20 sec, extension or amplification at 72°C for 30 sec (25 cycles, respectively), and elongation at 72°C for 5 min to stabilize the amplification product.

Reference meatball testing: The reference samples used in this study were meatballs made from beef and canine with known concentrations as shown in Table 1. Fresh beef and canine meat (CM) were washed and drained. The prepared meat (beef, beef-CM, CM) as much as 90% was mashed and mixed with wheat (10%), salt (0.01% wt/wt), and other herbs commonly used in making meatballs. The mixture was then formed into a ball and then boiled in boiling water until cooked. The DNA of reference meatball was extracted and then tested using real-time PCR. The condition of real-time PCR method used was according to the manufacturer's procedure given (Biotium Inc., Bio-Rad laboratories, C.A., USA). In reaction tube, 10 μL of EvaGreen, 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 analyzed using real-time PCR instrument (CFX96 Touch Real-Time PCR Detection System, Biorad USA). The real-time PCR procedure was as follows: pre-denaturation at 95°C for 3 min (1 cycle), denaturation at 95°C for 15 sec (25 cycles), annealing at 50,6°C for 20 sec and extension or amplification at 72°C for 30 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. The evaluation included linearity, efficiency, and sensitivity. Data were processed and analyzed using CFX Maestro™ software included in q-PCR instrument.

Validation of real-time PCR for quantitative analysis: Real time-PCR using CYTBCA3-kh was subjected to validation procedure according to Codex Allimentarius Commission (CAC/GL, 2010) by determining several characteristics performances including amplification efficiency, sensitivity expressed by limit of detection (LoD) and repeatability. The validated real time-PCR method was then used for identification of DNA in commercial meatballs samples.

Results

DNA extraction

DNA extraction was performed using phenol-chloroform-isoamyl alcohol method and the extracted DNAs were then subjected to electrophoresis using gel agarose for the identification of DNA integrity. Fig. 1 showed the gel agarose electropherogram of DNAs isolated from canine meat (CM), pork, wild-boar, chicken, beef, goat, rat and rabbit meats as well as those extracted from meatball samples containing canine CM-beef variations.

Table 2 exhibited the results of concentrations (yield) and the purity of DNAs extracted from several matrices.

Table 1: Composition of meatball reference samples containing beef (B) and Canine meat (CM)

No	Sample	CM (%)	Beef (%)
1	Beef meatball without Canine	0	100
2	Beef meatball + CM 0.1%	0.1	99.9
3	Beef meatball + CM 0.2%	0.2	99.8
4	Beef meatball + CM 0.3%	0.3	99.7
5	Beef meatball + CM 0.5%	0.5	99.5
6	Beef meatball + CM 0.7%	0.7	99.3
7	Beef meatball + CM 0.9%	0.9	99.1
8	Beef meatball + CM 1%	1	99
9	Beef meatball + CM 2%	2	98
10	Beef meatball + CM 3%	3	97
11	Beef meatball + CM 5%	5	95
12	Beef meatball + CM 10%	10	90
13	Beef meatball + CM 25%	25	75
14	Beef meatball + CM 50%	50	50
14	Canine meatball 100%	100	0

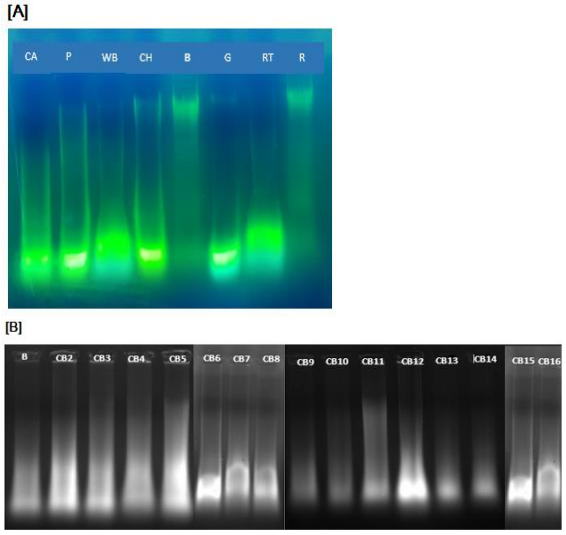

Fig. 1: The gel agarose electropherogram of DNAs isolated from Canine (CA), Pork (P), Wild-boar (WB), Chicken (CH), Beef (B), Goat (G), Rat (RT) and Rabbit (R) [**A**] and those extracted from meatball samples containing canine meat-beef variations (CB 2-16) [**B**]

DNAs extracted had ratio values approaching 1.7–2.0 with high yield indicated that DNAs are enough to be used for real-time PCR analysis.

Fig. 2 exhibited the amplification curve and melting analysis obtained using CYTBCA3-kh primer with DNA template extracted from fresh CM using different annealing temperatures. The temperature of 50.6°C offered the highest response. The designed primer was specific in which primer CYTBCA3-kh only amplify DNA extracted from canine meat (CM), as shown in Fig. 3.

Validation

Fig. 4 exhibited the amplification curve of real-time PCR using primer CYTBCA3-kh obtained during amplification of DNA extracted from fresh canine meat with different

Table 2: The purity and yield of DNAs extracted from fresh meat and those extracted from meatballs containing canine meat and beef with different concentrations

Samples	Concentration (ng/ μL)	Ratio (260/ 280)
Fresh meat		
Canine	2697.72	1.94
Pork	117.13	1.72
Wild-boar	3037.93	1.96
Chicken	3561.65	1.82
Beef	1897.80	1.50
Goat	128.82	1.81
Rattus	1730.86	1.87
Rabbit	2305.95	1.50
*Meatballs**		
Beef without Canine (0%)	399.68	1.29
Beef-Canine (0.1%)	583.35	1.64
Beef-Canine (0.3%)	783.29	1.61
Beef-Canine (0.5%)	3235.47	1.34
Beef-Canine (0.7%)	842.40	1.60
Beef-Canine (0.9%)	1390.81	1.44
Beef-Canine (1%)	898.24	1.65
Beef-Canine (2%)	678.37	1.54
Beef-Canine (3%)	531.44	1.74
Beef-Canine (5%)	2247.16	1.48
Beef-Canine (10%)	953.15	1.63
Beef-Canine (25%)	2395.33	1.68
Beef-Canine (50%)	1159.26	1.61
Beef-Canine (75%)	2930.50	1.47
Canine (100%)	1406.28	1.43

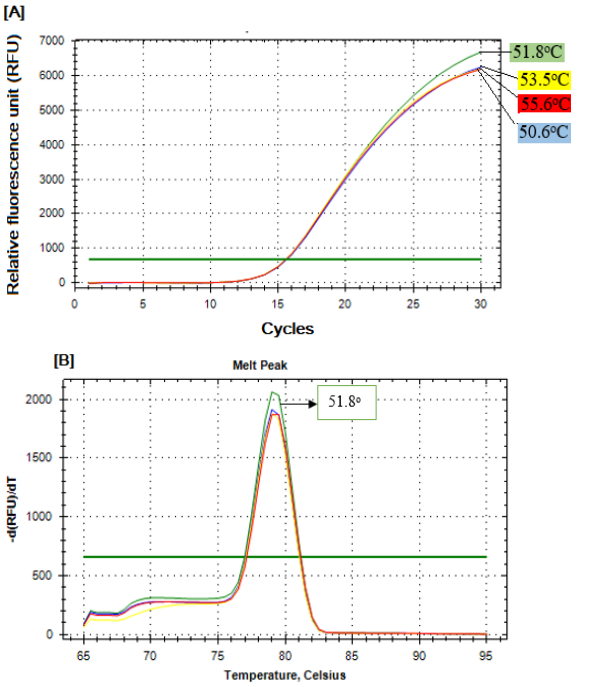

Fig. 2: The amplification curve [**A**] and melting peak analysis [**B**] of DNA from fresh tissue using primer CYTBCA3-kh with annealing temperature of 51.8

concentrations (50, 100, 500, 1000, 5000, 10000, and 50000 pg) for determining absolute limit of detection. The relative LoD value obtained was 50 pg DNA corresponding to 0.1% of CM in meatballs.

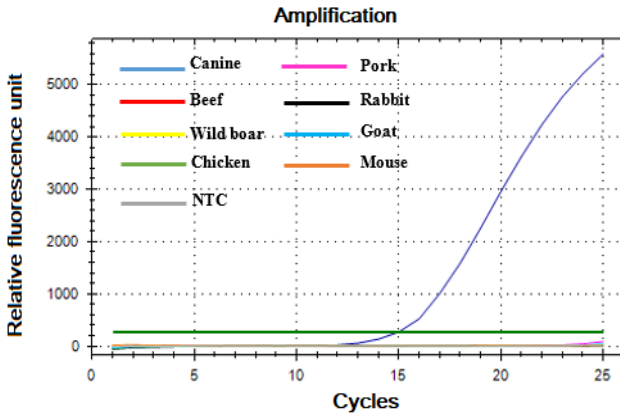

Fig. 3: The amplification curve of real-time PCR for specificity test of designed primer CYTBCA3-kh using DNA templates extracted from beef, pork, rabbit, goat, mouse, wild boar meats

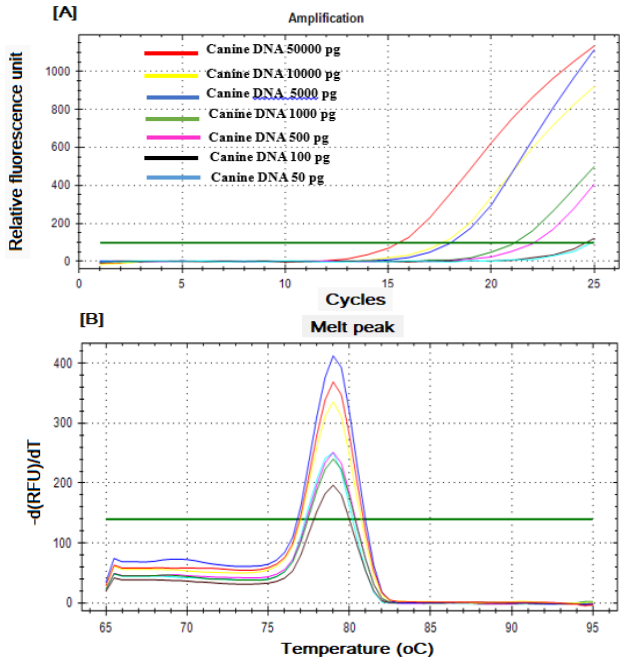

Fig. 4: The amplification curve of real-time PCR using primer CYTBCA3-kh [**A**] along with melting peak analysis [**B**] obtained during amplification of serial concentrations of DNA extracted from fresh dog meat for determining absolute limit of detection

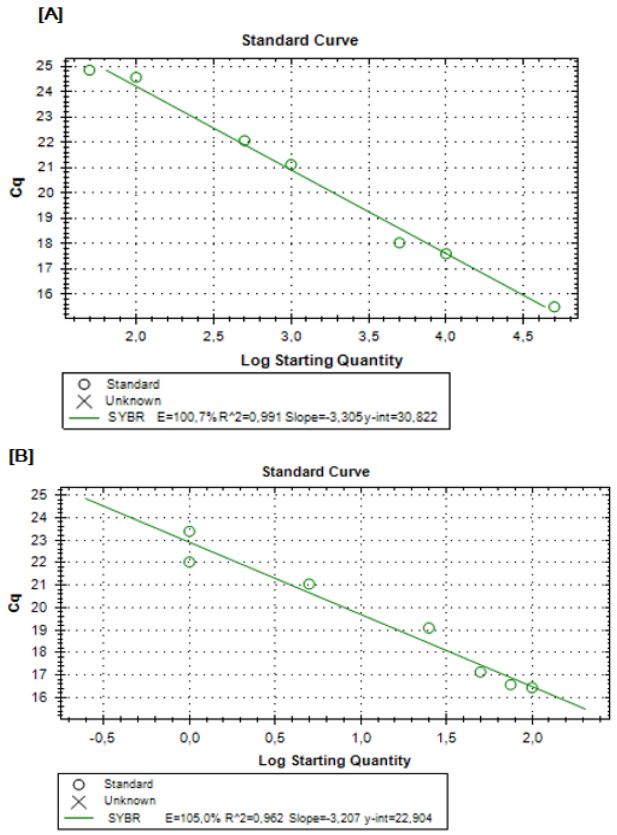

Fig. 5: Standard curves for linear relationship between quantification cycle (Cq) values (y-axis) and log of DNA concentrations (x-axis) extracted from fresh canine meat [**A**] and those extracted from meatballs containing canine meat used for calculating efficiency of amplification (**B**)

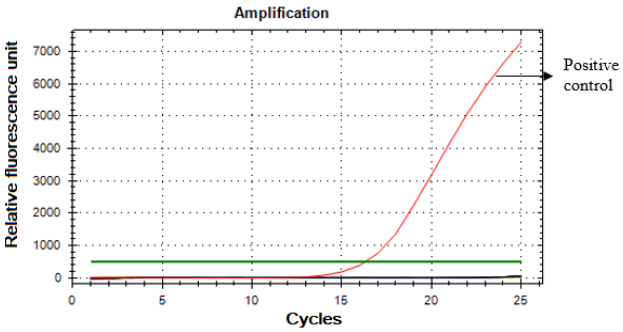

Fig. 6: The amplification curve of DNAs extracted from 15 commercial meatball samples along with positive control using primer CYTBCA3-kh

The relationship between quantification cycle (Cq) values (y-axis) and log of DNA concentrations (x-axis) extracted from fresh CM and those extracted from meatball containing CM was shown in Fig. 5. Good linear relationship existed with coefficient of determination (R^2) of 0.991, the slope of linear regression of -3.305, and y-intercept of 30.822 for DNAs extracted from fresh CM with efficiency value (E) of 100.7%. In addition, R^2 of 0.962, slope of -3.207 and y-intercept of 22.904 with E value of 105.0% were obtained for DNAs extracted from meatballs containing CM. The RSD values of Cq using DNAs extracted from fresh meat and meatballs were 0.0331% and 0.0302%, respectively.

The validated real-time PCR method was then applied for checking the presence of CM in commercial meatballs. No amplification for DNA templates isolated from the evaluated samples (Fig. 6), therefore, it could be stated that commercial samples evaluated did not contain CM and were considered as halal.

Discussion

The selection of target gene (mitochondrial cytochrome-b) was based on the results of the published data from our group concerning the identification of various DNAs extracted from non-halal meats (pork, canine meat, monkey meat, and wild boar meat) using specific primer targeting on Cyt-b gene (Ali *et al*. 2015; Guntarti *et al*. 2017; Arini *et al*. 2018; Rahayu *et al*. 2018b). The use of mitochondrial DNA of Cyt-b as targeted gene because almost all cells contain huge number copies of mitochondrial DNA and mutation rate offering good sensitivity with low detection limit. Besides, mitochondrial DNA are also easier to be isolated and to be purified for further steps of analysis (Kitpipit *et al*. 2014).

The successful analysis of meat species identification using real-time PCR is dependent to the yield and quality of DNAs extracted from fresh meat and those extracted from meatballs because DNA may be heterogeneous in composition due to processing. DNA must comply with minimum requirements in relation to (1) integrity, which determine the fraction of DNA that can be amplified by PCR, (2) yield in which DNA has to be in sufficient amount for analysis and (3) purity which plays a crucial role in DNA analysis because the presence of carbohydrates, proteins, and other metabolites can interfere during real-time PCR reactions which can be assessed based on the size distribution of fragmented DNA (Cravero *et al*. 2019). The DNAs have been successfully extracted from fresh meats and those extracted from meatballs containing CM and beef with no degradation observed. The extracted DNAs look smear as indicated by the presence of RNA, but it did not interfere in real-time PCR amplification. DNAs extracted had ratio values approaching 1.7–2.0 with high yield indicated that DNAs are enough to be used for real-time PCR analysis. Besides, the meatballs processing with heat do not cause DNAs to degrade.

The application of correct annealing temperature determined the capability of designed primer to amplify the DNA templates, and for this reason, several temperatures namely 50.6, 51.6, 53.5 and 55.6°C were tested. The temperature of 50.6°C offered the highest response expressed as relative fluorescence unit (RFU) among others and supported with melting curve analysis having single peak amplification. The main advantage of real-time PCR for species identification is related to its specificity. The designed primer was subjected to specificity test by applying it to DNA templates extracted from meats with different species, namely beef, pork, rabbit, goat, mouse, wild boar meat along with negative control or no template control (NCT). The amplification curve revealed that only DNA extracted from CM could be amplified with Cq of 15.1 indicating that CYTBCA3-kh primer was specific to CM.

The precision of real-time PCR was evaluated through repeatability test of quantification cycles (Cq) from six replicates. The DNA concentration used for real-time PCR amplification were 1000 pg/µL extracted from fresh canine meat and meatballs containing 100% canine meat. The relative standard deviation (RSD) was used for evaluation of repeatability. The RSD values of Cq using DNAs extracted from fresh meat and meatballs were 0.0331% and 0.0302%, respectively. Codex Allimentarius Commission (C.A.S., 2010) required that PCR method was considered as precise if maximum RSD values of 25% were obtained. Therefore, real-time PCR using designed primer was precise enough and the random error occurring during analysis can be negligible.

The sensitivity of primer CYTBCA3-kh coupled with real-time PCR analysis for amplification of DNA template was expressed with the minimum DNA amount could be amplified with relative fluorescence unit of 100 (Forootan *et al*. 2017), known as value of limit of detection (LoD). There are two types of LoD values, namely absolute LoD value obtained by amplification of DNA extracted from fresh dog meat from serial dilution to obtain DNA with different concentration and relative LoD obtained by amplifying DNA extracted from meatball containing 100% dog meat. Canine DNA extracted from fresh meat could be amplified up to 50 pg, while at below 50 pg, RFU obtained was lower than 100, therefore relative LoD value obtained was 50 pg DNA. In addition, relative LoD of DNA CM obtained was also 50 pg corresponding to 0.1% of CM in meatballs. Therefore, meatballs containing 0.1% CM could be detected using developed real-time PCR method with CYTBCA3-kh primer.

Good linear relationship existed with coefficient of determination (R^2) of 0.991, the slope of linear regression of -3.305, and y-intercept of 30.822 for DNAs extracted from fresh CM with efficiency value (E) of 100.7% (Fig. 5A), and R^2 of 0.962, slope of -3.207 and y-intercept of 22.904 with E value of 105.0% (Fig. 5B) for DNAs extracted from meatballs containing CM. Codex Allimentarius Commission (CAS, 2010) declared the acceptance criteria for quantitative PCR assay is R2 of ≥0.98 and E value of 90–110%. Therefore, the E values met the requirements, while R^2 values fit for DNAs extracted from fresh CM, but R^2 value obtained from meatballs containing CM was smaller than that required by CAS. The validated real-time PCR method was then applied for checking the presence of CM in commercial meatballs. There were 15 samples along with positive control evaluated. No amplification for DNA templates isolated from the evaluated samples, therefore, it could be indicated that commercial samples evaluated did not contain CM and were considered as halal.

Conclusion

The designed primer (CYTBCA3-kh) was specific to DNA extracted from CM and that extracted from meatballs containing CM using optimized annealing temperature of 50.6°C. The validation parameters of real-time PCR met the

required criteria according to Codex Allimentarius Commission. The validated real-time PCR using CYTBCA3-kh primer could successfully identify CM in meatball formulation for halal authentication analysis and potential to be used as standard official method.

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