TaqMan real-time polymerase chain reaction for the determination of pork adulteration in meat nuggets

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Summary

A TaqMan real-time polymerase chain reaction was developed for the determination of pork adulteration in nuggets. The assay combined species-specific primers and TaqMan probe to target a 109 bp fragment of swine cytochrome b gene. Specificity test with 10 ng DNA of 11 different meat species yielded a quantification cycle (*Cq*) between 16.9 and 17.1 for pork, and negative results for other meats. Model experiment using chicken nuggets spiked with pork showed that the assay can quantify 100–0.01% of pork adulteration with a linear correlation (R^2 of 0.998, PCR efficiency of 91.1%, and relative error ≤ 5%). A plot of actual value against real-time PCR-predicted value yielded R^2 of 0.999, and a very small (0.242) root mean square error of calibration. A strong correlation was found between the partial least square-predicted values and the values determined by real-time PCR. Random analysis of nuggets from pork, beef, chicken, mutton and chevon yielded *Cq* values between 18.2 and 18.6 for pork nuggets, and negative results for other meat nuggets. Finally, analysis of 27 commercial nuggets from each of the five common meat species revealed the presence of pork in 100%, 3.7%, 7.4%, 3.7% and 0% nuggets of pork, beef, mutton, chevon and chicken, respectively.

Keywords

fortified chicken nuggets; TaqMan; Halal; Kosher; authentication; meat emulsion

Verification of declared components in commercial meat products not only helps consumers to make a well-defined purchase decision, but also protects health, religious belief, and endangered wildlife [1–3]. As minced meat is being increasingly used as an additive in most of the modern and fortified packaged foods [4], verification of meat species in processed food should gain consumer trusts, prevent unfair market competition, promote fair trade, protect endangered animal species and restore biodiversity in natural habitats [2–4]. According to European law (Regulation (EC) No. 178/2002) on food safety, each stakeholder in a food supply chain must be able to identify all raw materials used in the preparation of food products [5].

The total turnover of global Halal food market stood at USD 641 billion in 2010 and is expected to be crossing USD 661 billion by 2011 [6, 7]. The demand for ready-made Halal foods, such as burgers, pizza, sandwiches, hot dog, soups, cookies, candies and creams, is rapidly expanding to commensurate the needs of the on-growing number of working Muslim population [6]. Since the prices of Halal and Kosher foods are comparatively higher because of their special processing requirements, fraudulent labelling of these brands frequently occurs [1, 4, 8].

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Meat nugget is a type of fast food that is popular in all parts of the world [9]. It can be prepared from deboned meats, vegetable proteins, gum, egg and a fair portion of dietary fibres [9–11]. Replacement of valuable ingredients for the cheaper ones and the addition of ingredients to improve appearance are the most common forms of adulteration in food industry [4, 9, 12]. Pork is a potential adulterant of Halal and Kosher foods, such as nugget formulations, because of its lower price, better textural stability and easier availability [4]. Several reports have been published on detection of pork adulteration in poultry and cattle meat products [2, 4, 8]. However, the presence of pork or any of its derivatives in Halal and Kosher food products is totally banned by the religions of Islam and Judaism [4].

A number of analytical methods have been developed for identification of pork and/or lard, such as electronic nose and gas chromatography-mass spectrometry [13], Fourier transform infrared spectroscopy [14], enzyme-linked immunosorbent assay [15], PCR with electrophoresis [12], PCR-RFLP (restriction fragment length polymorphism) [4], real-time PCR with TaqMan probe [16–18], real-time PCR molecular beacon [19], SYBR green real-time PCR [8] and nanoparticle sensors coupled with optical or fluorescence spectroscopy [20–22]. Among these methods, real-time PCR assays combined with species-specific primers and TaqMan probe are particularly promising because they provide enhanced specificity and reliability of the assay [2, 16–18]. Both simplex [18] and multiplex [17] real-time PCR assays with TaqMan probes have been proposed for meat species identification. Multiplex PCR assays allow simultaneous identification of several species using a single PCR assay, reducing both the cost and time [17]. However, they need comparatively longer and length-variable DNA templates for different species [16, 17]. As the longer DNA templates are not stable in the harsh conditions of food-processing, and length variable templates entail variable sensitivities for different species [2, 4, 18], multiplex PCR assays are not suitable for the analysis of processed foods.

On the other hand, simplex real-time PCR assays are comparatively easier to design and ensure more sensitive and robust quantitative detection of species in raw [19] and processed [18] states. Thus, a real-time PCR assay targeting multicopy genes, such as mitochondrial genes, with a shorter amplicon, is of interest as it ensures available targets even in degraded samples, significantly increasing the assay sensitivity and reliability [2, 18].

In this paper, we combined the use of species-specific primers and a TaqMan probe to selectively amplify and detect a short fragment (109 bp) of swine cytochrome b (cytb) gene of mitochondrial DNA by a real-time PCR assay. To the best of our knowledge, it was the shortest template so far utilized in a real-time PCR assay to trace pork derivatives in commercial meat products. In this assay, we used a TaqMan probe of 31 nucleotides to increase both the annealing temperature and coverage of interspecies polymorphism. Increased annealing temperature and polymorphism provide improved specificity [4, 23]. However, the use of longer probe increases intermolecular distance between the fluorophore and quencher in the unhybridized state, increasing the background signal at detection [19]. In this work, the signal-to-noise ratio of the TaqMan probe was significantly increased by introducing two quenchers, ZEN in the middle and Iowa Black at the end (both produced by Integrated DNA Technologies, Coralville, Iowa, USA). The accuracy and precision of the method was tested in food mixtures with various percentages of spiked pork in ready-to-eat chicken nuggets. The real-time PCR results were further compared with the partial least square (PLS) model-predicted values. Finally, the suitability of the assay to determine pork in commercial nuggets was verified by analysing 27 commercial nuggets produced with five common meat species.

MATERIALS AND METHODS

Sample collections

The fresh raw muscle tissue of 6 meat-providing land animals (pork/pig: *Sus scrofa,* beef/cow: *Bos taurus*, mutton/sheep: *Ovis aries*, chevon/goat: *Capra hircus*, venison/deer: *Cervus nippon* and chicken/hen: *Gallus gallus*) and 5 aquatic species (cichlid: *Crenicichla minuano*, shad: *Alosa sapidissima,* shrimp: *Gadus morhua*, tuna: *Thunnus orientalis* and cuttlefish*: Sepia officinalis*) were procured in triplicates on three different days from Pasar Borong, Selangor, Malaysia. The identity of meat species was confirmed by the veterinary and fisheries experts from the Department of Animal Sciences in the Putra University, Serdang, Malaysia. The samples were cut into small pieces and stored frozen at –20 °C until use to prevent enzymatic degradation of DNA.

Commercial nuggets produced from chicken, pork, beef, mutton and chevon were purchased from three different superstores of Kuala Lumpur and Selangor, Malaysia on three different days in triplicates.

Preparation of chicken nuggets

Freshly slaughtered chicken and pork were obtained from the slaughterhouse of the Department of Animal Sciences (Putra University) on three different days. The meat specimens were chilled overnight at 4 °C and then manually deboned and minced. Muscles were taken from skeletal muscle (95%), intestine (2.5%), liver (1%), heart (0.5%), kidney (0.5%) and 0.5% skin to produce the tissue composition of a typical livestock meat. Binary meat mixtures were prepared by spiking 0%, 0.01%, 0.1%, 1%, 10% and 100% (w/w) pork to chicken meat in a 250 g portion of each spiking level. The added mixtures were then minced twice through a 4 mm plate in a meat mincer (Sin Huat Hin, Seremban, Malaysia). To a 200 g portion of each meat mixture, 10 g chilled water, 10 g soybean oil, 10 g textured soya protein, 15 g finely chopped raw carrot, 5 g refined wheat flour, 10 g whole egg liquid, 3 g spice mix, 5 g condiments (onion and garlic paste), 2 g sodium chloride, 1 g sugar, 0.4 g tetrasodium pyrophosphate and 25 mg sodium nitrite were added [10, 11]. An emulsion of each mixture was prepared by blending the above mixtures. The emulsion was manually filled in stainless steel molds and the lid was tightly closed. The molds were stream-cooked at an internal temperature of 80–85 °C for 15 min [24]. After the thermal process, the meat blocks were cooled down to room temperature and the nuggets of uniform size were shaped.

DNA extraction

Total DNA extraction was carried out from 5 mg of finely chopped raw muscle tissue of each species using MasterPure DNA Purification Kit (Epicenter Biotechnologies, Madison, Wisconsin, USA) according to the protocol of Epicenter Biotechnologies [4]. For commercial samples and prepared nuggets, 1 ml of cell and tissue lysis solution (Epicenter Biotechnologies) was added to a 100 mg portion of finely chopped specimen and was incubated in a shaking water bath at 65 °C for 12 h. The subsequent steps of the extraction protocol were performed as previously described [4]. The purity and content of extracted DNA was determined on the basis of spectrophotometric absorbance ($A_{260/280}$ and A_{260nm}; Biophotometer, Eppendorf, Hamburg, Germany).

Primer and probe design

A pair of primers (SwcytbF and SwcytbR) targeting the 109 bp fragment of swine cytb gene was designed by publicly available primer3Plus software (SBGrid Software Consortium, Boston, Massachusetts, USA) using a 27 nucleotide *Alu*I-cut fragment (429–455 bp) of *S. scrofa* cytb gene (GenBank No. *AF*034253.1 in NCBI database; National Center for Biotechnology Information, Bethesda, Maryland, USA) as an internal oligonucleotide. A 31 nucleotide swine TaqMan probe (SwcytbTqM), containing the 27 nucleotide *Alu*I-cut fragment within, was designed by tagging 6-carboxyfluoresceine (FAM) and 3-Iowa Black FQ (IABkFQ) at the 5' and 3' ends, respectively. For endogenous control, eukaryotic 18S rRNA specific primers (Eu18SrRNAF and Eu18SrRNAR) and TaqMan probe (Eu18SrRNATqM) described by Rojas et al. [18] were used. To increase the signal-to-noise ratio, a second quencher known as ZEN (Integrated DNA Technologies) was introduced at position 10 of both TaqMan probes. All the probes and primers were purchased from Integrated DNA Technologies and are shown in Tab. 1 along with the amplicon sequences.

Tab. 1. Primer, probe and amplicon sequences used in this study.

Name	Sequence (5'-3')
*Sw*Cytb*F*	TCC TGC CCT GAG GAC AAA TA
*Sw*Cytb*R*	AAG CCC CCT CAG ATT CAT TC
*Sw*Cytb*TqM*	6-FAM/AGC TAC GGT/ZEN/CAT CAC AAA TCT ACT ATC AGC T/3IABkFQ
Sw-amplicon (109 bp)	TCC TGC CCT GAG GAC AAA TAT CAT TCT GAG G*AG CTA CGG TCA TCA CAA ATC TAC TAT CAG*CTA TCC CTT ATA TCG GAA CAG ACC TCG TAG AAT GAA TCT GAG GGG GCT T
*Eu*18SrRNA*F*	GGT AGT GAC GAA AAA TAA CAA TAC AGG AC
*Eu*18SrRNA*R*	ATA CGC TAT TGG AGC TGG AAT TAC C
*Eu*18SrRNA*TqM*	6-FAM/AAG TGG ACT/ZEN/CAT TCC AAT TAC AGG GCC T/3IABkFQ
Eu-amplicon (141 bp)	GGT AGT GAC GAA AAA TAA CAA TAC AGG ACT CTT TCG AGG CCC TGT AAT TGG AAT GAG TCCACT TTA AAT CCT TCC GCG AGG ATC CAT TGG AGG GCA AGT CTG GTG CCA GCA GCC GCG GTA ATT CCA G*CT CCA ATA GCN TAT

* – *Alu*I restriction sites; *Sw* – swine; *Eu* – eukaryotic; *F* – forward primer; *R* – reverse primer; *TqM* – TaqMan probe.

Real-time PCR analysis

The real-time PCR assay was carried out in an Eppendorf Mastercycler ep-realplex machine (Eppendorf, Hamburg, Germany) with 20 μl reaction mixture consisting of 1x SsoFast probe supermix (Bio-Rad, Hercules, California, USA), 300 nmol.l^{-1} of each primer, 200 nmol.l^{-1} of TaqMan probe and 10 ng of DNA. Required dilution was performed using sterile deionized water and separate tubes were used for endogenous control and swine-specific assays. A two-step amplification programme consisted of pre-denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 61 °C for 20 s. Each sample and endogenous control was run in triplicates on three different days by three independent analysts.

Construction of standard curve and target quantification

A standard curve was constructed by plotting normalized quantification cycle (*Cq*) values against the logarithm of the content of starting pork DNA in the calibration set of different chicken nuggets. The amount of unknown set was quantified by interpolating the normalized *Cq* values in the standard curve [18, 23]. *Cq* of the pork-specific system (*CqPS*) was normalized according to Rojas et al. [18] using the equation given below:

$$CqNPS = CqEp \times CqSP/CqEB \quad (1)$$

where *CqNPS* is the normalized *Cq* value of the sample with pork-specific PCR system, *CqEp* is the average *Cq* value of 10 ng pork DNA from nugget composition in endogenous PCR system, and *CqEB* is the *Cq* value of the specific nugget sample with the endogenous PCR system.

However, validation experiment with various amount of spiked pork did not show any significant changes in the *Cq* values of the endogenous system at $P \leq 0.05$. Thus, it was assumed that $CqEP = CqEB$. Thus, the simplified form of the above equation was derived as

$$CqNPS = CqSP \quad (2)$$

meaning that there was no statistically significant difference between the normalized *Cq* and the raw *Cq* of the pork-specific system in chicken nugget formulations.

Statistical analysis and validation

Partial least square analysis and Anova test were performed by Minitab 14 software (Minitab, State College, Pensylvannia, USA). The "leave-one-out" cross validation method was used to verify the calibration model. The values of root mean square error of calibration (*RMSEC*) and coefficient of correlation (R^2) were used as the validation parameters for the calibration.

RESULTS AND DISCUSSION

Real-time PCR system

As a target for real-time PCR, a 109 bp fragment of swine mitochondrial cytb gene was chosen because of its suitable length that can survive the harsh treatments of food processing [2–4]. Cytb gene has long been a target for meat species analysis due to its multiple copy number, high rate of evolution, polymorphic intra- and inter-species features, and additional protection of DNA by the mitochondrial membrane [4]. The designed primers and TaqMan probe were verified by NCBI Blast analysis (National Center for Biotechnology Information) and ClustalW alignment analysis (Conway Institute of Biomolecular & Biomedical Research, University College Dublin, Dublin, Ireland) to ensure porcine specificity. A comparatively longer TaqMan probe (31 nucleotide) was used in the present work to ensure better specificity by a higher melting temperature. Higher annealing temperature reduces non-specific amplification and thus increases assay specificity [4, 19]. In order to improve quenching of the longer FAM-labelled probe, ZEN quencher was used at position 10 in addition to 3'-quencher (Iowa Black).

A eukaryotic primer set and TaqMan probe that specifically amplify and detect a conserved 141 bp fragment of 18S rRNA was selected as endogenous control [18]. The use of endogenous control in real-time PCR assays serves several purposes:

1. It provides information about the total PCR-amplifiable DNA in the sample to take into account the potential factors, such as PCR inhibition, template degradation and quality of DNA recovered from the source sample that might affect PCR amplification;
2. It traces the presence of DNA that may not be amplified by the species-specific detectors (primers and probe);
3. It facilitates a comparison of species-specific and endogenous control signals to reduce inaccuracies caused by the standards and the unknowns [18].

Specificity test

The specificity of the porcine-specific TaqMan real-time PCR assay was assessed with 10 ng of DNA extracted from the fresh muscle tissues of

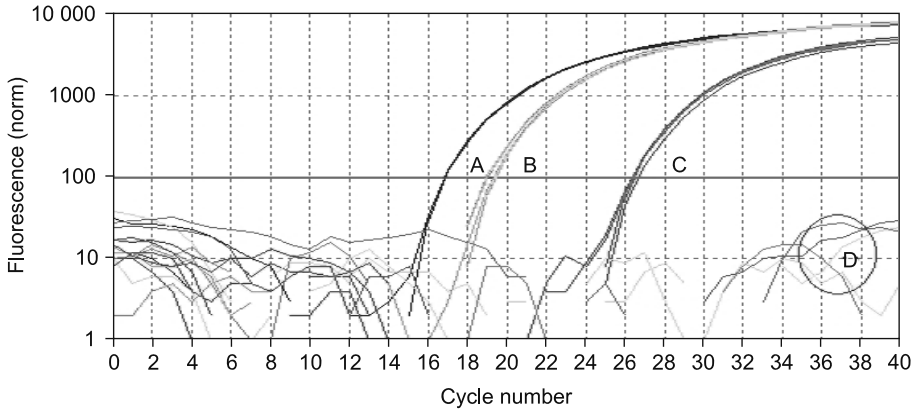

Fig. 1. Real-time PCR records of the porcine-specific and endogenous control PCR systems for land animal and aquatic species.

A – porcine-specific, B – land animal species, C – aquatic species, D – no-template controls (NTC) of both systems and non-amplified PCR-products of porcine-specific system for other species.

major meat-providing land animals (cow, chicken, lamb, goat, deer and pig) and aquatic species (shrimp, tuna, cuttlefish, cichlid and shad) changing sources and analysts on three different days in triplicates. Positive control was amplified in different tubes using the similar experimental set up by changing primers and probe to evaluate the quality of total amplifiable eukaryotic DNA in each tube [18]. The amplification profiles are shown in Fig. 1. *Cq* values between 16.93 ± 0.15 and 17.11 ± 0.28 (Fig. 1, curve A) were obtained from porcine DNA from a total of 9 replicates (3 replicates from each of 3 different extractions) using the porcine-specific PCR system. Other species provided only the background fluorescence (Fig. 1, curve D) within 40 cycles of amplification, demonstrating that the assay was specific for swine cytb gene.

Endogenous control showed two distinct profiles of *Cq* values, targeting animal and aquatic species. The mean of endogenous *Cq* values (average of three determinations) of animal species were between 19.11 ± 0.2 and 19.71 ± 0.14 (Fig. 1, curve B) and those of fish species were 26.19 ± 0.09 and 26.94 ± 0.02 (Fig. 1, curve C). Alignment analysis of the 18S rRNA genes of the above species by ClustalW alignment program revealed 100% matching of the primers and probe with the animal species. 18S rRNA of all fish species were not available in NCBI database. We aligned the primers and probe with the 18S rRNA gene of shrimp (*Gadus morhua AF518205.1*). Both endogenous primers showed 100% matching. However, the probe alignment demonstrated four nucleotide mismatches. Smith et al. [23] studied the effect of different degrees of mismatches on the primer- and probe-binding regions of different-length amplicons. They observed that mismatches at the probe-binding region significantly increased the *Cq* values, greatly reducing the real-time PCR efficiency.

Alignment analysis of the porcine-specific primers and probe demonstrated 100% matching only with swine cytb gene (*S. crofa* AF034253.1) while all other species (*B. taurus* EU807948.1, *G. gallus* EU839454.1, *O. aries* EU365990.1, *C. hircus* EU130780.1, *C. nippon* EF139156.1, *G. morhua* AM489716.1, *T. orientalis* AM989973.1, *S. officinalis* EF423081.1, *C. minuano* GQ199921.1 and *A. sapidissima* EU552616.1) had a huge number of mismatches. Earlier studies demonstrated that presence of single mismatch base in the primer- and probe-binding region of the template does not significantly affect the PCR efficiency. However, combined effects of primer and probe mismatches strongly reduced the PCR efficiency causing amplification failure in PCR process [23]. Moreover, primer annealing at high temperature increases specificity and eliminates non-specific primer hybridization [4]. Therefore, realization of specific PCR product from the perfectly matched swine cytb gene at a high annealing temperature (61 °C) was justifiable and thermodynamically sound.

PCR efficiency and limit of detection

Usually, 10-fold serial dilutions of pure DNA are used to construct a standard curve in order to determine the limit of detection (*LOD*) and efficiency of a PCR system [19]. Binary mixtures of two different DNA species may also be utilized for this purpose [18]. However, they do not re-

flect the effects of complex matrices often found in processed food [4] and, consequently, cannot be used for accurate quantification of the analyte in commercial products [18]. To eliminate the above limitations, chicken nuggets with various percentages of spiked pork were prepared simulating the common recipe of commercial nuggets [10, 11, 24]. As the number of mitochondria and mitochondrial genes are tissue-dependent [2, 4], finely chopped deboned meats of different tissues (95% skeletal muscle, 2.5% intestine, 1% liver, 0.5% heart, 0.5% kidney, and 0.5% skin) were used for the preparation of nugget meats.

The amplification profiles of 10 ng DNA extracted from chicken nuggets with 100%, 10%, 1%, 0.1%, 0.01%, and 0% of spiked pork are shown in Fig. 2. Strong fluorescence signal was obtained from all tubes containing pork DNA with *Cq* values ranging from 18.2 (10 ng = 100%) to 32.7 (0.001 ng = 0.01%) (Tab. 2), depending on the level of spiked pork in the respective nugget formulation. The endogenous control showed

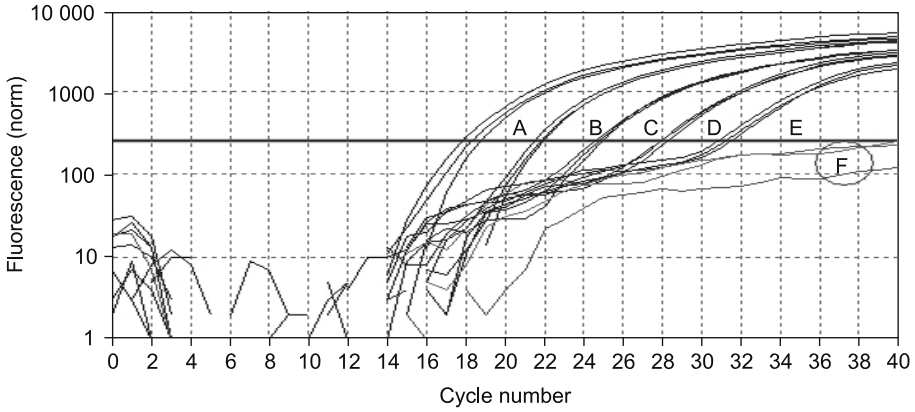

Fig. 2. Real-time PCR records obtained for chicken nuggets with 0.01–100% (w/w) of spiked pork by the porcine-specific PCR system.

A, B, C, D, E – letters represent amplification curves for samples containing 100%, 10%, 1%, 0.1% and 0.01% of spiked pork, respectively; F – NTC and 0% pork.

Tab. 2. Number of quantification cycles at which fluorescence was first detected from 10 ng DNA of pork-spiked chicken nuggets.

Pork [%]	Day	Mean number of quantification cycles		Number of replicates	Probability [%]
		Porcine-specific	Endogenous		
100	Day 1	18.19 ± 0.18^{a}	20.19 ± 0.23^{a,b}	3/3	100
	Day 2	18.53 ± 0.11^{a}	20.65 ± 0.07^{a,b}	3/3	100
	Day 3	18.28 ± 0.11^{a}	20.35 ± 0.09^{a,b}	3/3	100
10	Day 1	21.56 ± 0.06^{b}	20.33 ± 0.07^{a,b}	3/3	100
	Day 2	21.84 ± 0.10^{b}	20.59 ± 0.14^{a}	3/3	100
	Day 3	21.76 ± 0.12^{b}	20.49 ± 0.13^{a}	3/3	100
1	Day 1	25.13 ± 0.39^{c}	20.21 ± 0.15^{a,b}	3/3	100
	Day 2	25.08 ± 0.15^{c}	20.00 ± 0.18^{a,b}	3/3	100
	Day 3	25.34 ± 0.10^{c}	20.45 ± 0.09^{a}	3/3	100
0.1	Day 1	29.13 ± 0.27^{d}	19.86 ± 0.51^{b}	3/3	100
	Day 2	28.73 ± 0.06^{d}	20.44 ± 0.15^{a}	3/3	100
	Day 3	28.99 ± 0.14^{d}	20.6 ± 0.16^{a}	3/3	100
0.01	Day 1	32.70 ± 0.21^{f}	20.66 ± 0.14^{a}	3/3	100
	Day 2	32.48 ± 0.09^{f}	20.47 ± 0.12^{a}	3/3	100
	Day3	32.33 ± 0.10^{f}	19.94 ± 0.18^{b}	3/3	100

Means within the same columns followed by the same subscript letter are not significantly different at $P \leq 0.05$.

Tab. 3. Comparison of actual values with real-time PCR results for the determination of pork in chicken nuggets.

Sample	Actual [ng]	Found by PLS [ng]	Recovery [%]	Found by PCR [ng]	Recovery [%]
1	10	10.16	101.6	10.2	102
2	0.001	0.00096	96.0	0.000986	98.6
3	0.1	0.0972	97.2	0.9892	98.9
4	1	0.982	98.2	0.985	98.5
5	0.01	0.00956	95.6	0.00976	97.6
6	1	1.032	103.2	1.043	104.3
7	0.1	0.096	96.0	0.0978	97.8
8	0.001	0.00105	105.0	0.00104	104.0
9	1	0.993	99.3	1.018	101.8
10	0.01	0.0104	104.0	0.0103	102.0
11	10	9.908	99.1	9.793	97.9
12	0.01	0.0096	96.0	0.0098	98.0
13	0.1	0.0957	95.7	0.0965	96.5
14	0.001	0.00103	103.0	0.00104	104.0
15	10	9.691	96.9	9.802	98.0

Average values of three determinations are presented.

a constant level of amplification with a mean of *Cq* values between 20.2 and 20.7 (Tab. 2), showing no significant dependency of endogenous *Cq* on the level of pork adulterant. ANOVA test revealed no significant difference in endogenous *Cq* values at 95% confidence level. This agreed with theory as the endogenous control targeted a conserved region in eukaryotic 18SrRNA gene, and the pork and chicken 18S rRNA were 100% identical as found by alignment analysis (*S. scrofa* AM711871.1 and *G. gallus* FM165414.1).

A total of 45 replicates (3 on 3 different days for 5 different chicken nuggets) containing 0.01–100% of spiked pork were tested and three outliers (19.86 ± 0.51, 19.94 ± 0.18, and 20.00 ± 0.18) were identified. When outliers were omitted, no significant differences in endogenous *Cq* values were observed at $P \leq 0.05$ (Tab. 3). Even when outliers were included, no significant difference was obvious at $P \leq 0.1$. These results justified elimination of *Cq* normalization at the construction of standard curve [18]. Thus, raw *Cq* values of the pork-specific PCR system obtained from 9 replicates of each nugget formulation (Tab. 3) were plotted against the logarithmic values of porcine DNA content in each preparations. A good linear regression was obtained with a high correlation coefficient ($R^2 = 0.998$) and a slope of –3.557 (Fig. 3). A calculation of PCR efficiency (E) using the formula

$$E = [10^{(-1/\text{slope})} - 1] \quad (3)$$

yielded an efficiency of 91.1% [19]. Generally, real-time PCR efficiency varies between 90% and 110%, despite a high linearity ($R^2 = 0.99$). Based on this calculation, Rodriguez et al. [25] obtained a PCR efficiency of 64.8% and 68.9% in raw and autoclaved pork-beef binary mixture. They used a 411 bp fragment of porcine 12S rRNA gene as amplicon and achieved an *LOD* of 0.1% pork in pork-beef binary mixtures. Using

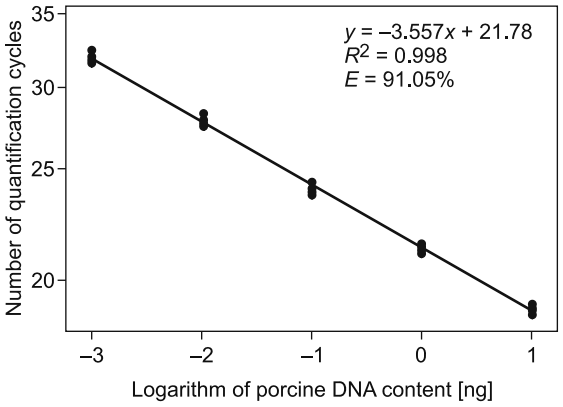

Fig. 3. Standard curve for the porcine-specific real-time PCR obtained with chicken nuggets containing 100% (10 ng), 10% (1 ng), 1% (0.1 ng), 0.1% (0.01 ng) and 0.01% (0.001 ng) of spiked pork.

The curve was generated using a total of 9 replicates (3 replicates of 3 different preparations on 3 different days with a change of sources and analysts) of each contamination level, depicted by points.

a 119 bp amplicon and molecular beacon probe of porcine cytb gene, Yusop et al. [19] achieved 96% efficiency with pure DNA and 0.1% *LOD* in binary meat mixtures. However, they did not test their PCR system in a complex background of processed meat products, which often contain different ingredients, and did not test different degradative treatments, such as heating, boiling, frying, roasting or pressuring. To calculate *LOD*, they considered also $Cq > 35$, which is not reliable with ≥ 95% certainty [18, 26].

The present assay optimized the PCR system in a complex background of fibre-fortified chicken nugget [10], considering the effects of all potential ingredients. The tissue composition of typical nugget meat was also carefully considered. A very low *LOD* of 0.01% (w/w) pork at Cq < 33 and PCR efficiency of 91.05% were achieved. Earlier studies demonstrated that smaller-sized amplicons are more stable, being amplified more efficiently than the longer ones [2–3, 20, 26–27, 32]. Thus, the high PCR efficiency and low *LOD* of the present work can be partly attributed to the smaller-sized DNA target (109 bp). Another reason might have been the use of a double-quenched TaqMan probe that generated high signal-to-noise ratio in the PCR amplification process.

Calibration and validation by partial least square model

Partial least square (*PLS*) is a statistical multivariate calibration method based on factor analysis [27]. It simultaneously decomposes both the content matrix and signal matrix of a mixture of standard solutions in a calibration set into latent variables that are not considered in ordinary regression analysis [28]. *PLS* and principal component analysis (*PCA*) only need to know the content of the analyte of interest in the calibration samples and it is unnecessary to know the content of other analytes [28]. However, *PLS* is superior to *PCA* because *PLS* decomposes both the content and signal matrices, and *PCA* decomposes only the signal matrix to derive the latent variables (factors) [28]. In this paper, we selected *PLS* as a data processing tool.

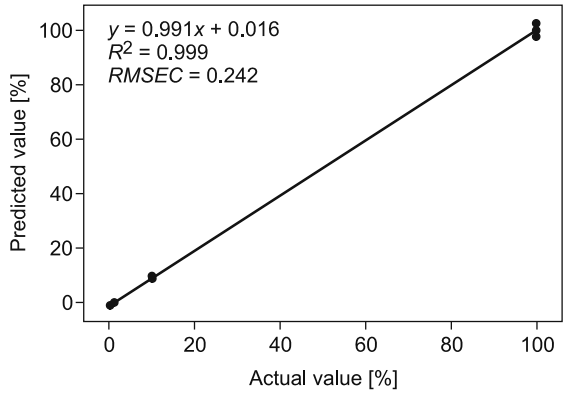

Fig. 4. Relationship between the actual and real-time PCR-predicted values (% w/w) of pork DNA in chicken nuggets, containing 0.01%, 0.1%, 1%, 10% and 100% of spiked pork.

Two different sets of standard chicken nuggets containing 0.01–100% of adulterated pork were designed for the calibration and validation models. The quantification of the pork content of calibration set was performed by the pork-specific real-time PCR, using triplicates of each specimen. Real-time PCR predicted value (*y* axis) was plotted against the actual value (*x* axis) and a calibration model was established with a very high correlation coefficient ($R^2 = 0.999$) and very low root mean square error of calibration (*RMSEC* = 0.242) (Fig. 4).While R^2 value provides information about the closeness of relationship; *RMSEC* describes the degree of uncertainty. The closer the R^2 value to unity the better the relationship, and the smaller the *RMSEC* the better the calibration model [16].

Selection of optimum number of factors in PLS model

Selection of the optimum number of latent factors in *PLS* algorithm is necessary to estimate the complexity of the system without overfitting the content data. To accomplish this goal, cross-validation of the calibration model was performed leaving one sample out at a time [29]. Given a set of randomly selected 15 calibration samples (Tab. 3), *PLS* calibration was performed on 15–1 = 14 samples, and the content of the sample left out was predicted using the new regression model. This process was repeated for a total of 15 times until each sample had been left out once. The content predicted for each sample was then compared with the known content of this reference sample. The sum of the squared prediction errors for all calibration samples (prediction error sum of squares or *PRESS*) is a measure of how well a *PLS* model fits the content data [14, 29]. *PRESS* is calculated in the same manner each time a new factor is added to *PLS* model. Usually, the following equation is used to determine *PRESS*,

$$PRESS = \sum_{i=1}^{n} \left(\hat{C}i + Ci\right) \quad (4)$$

where n is the number of samples in the prediction set, C_i is the actual content in the i^{th} sample, and $\hat{C}_i$ is the estimated value.

One of the reasonable ways to determine the optimum number of factors would be the factors that yield minimum *PRESS* [29]. Usually, *F*-statistic is used to make a significant determination in order to find a model with the fewest number of factors. In the present work, *PRESS* informed that the optimum factor number was 8 (Fig. 5), meaning that *RMSEC* reached a stable value after minimally eight factors [14]. This confirmed that *Cq* values used for developing *PLS* model for the quantification of pork DNA in chicken nugget formulations had significant correlation with the target DNA content. The *Z*-pattern of the *PRESS* curve further reflected the reduced sensitivity of the methods at extreme target contents, meaning that there is a scarcity of available targets at very low contents and a saturation of substrate at very high contents.

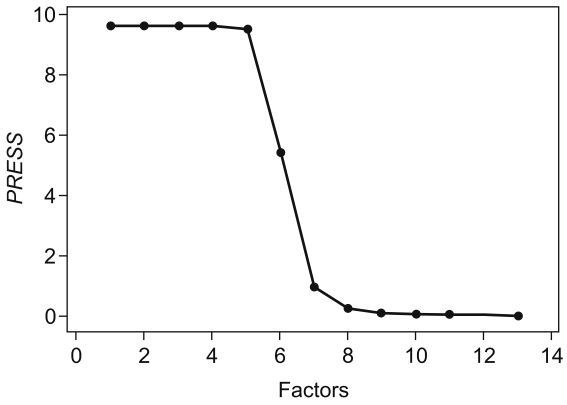

Fig. 5. *PRESS* values obtained from PLS mode for different numbers of latent factors in chicken nuggets.

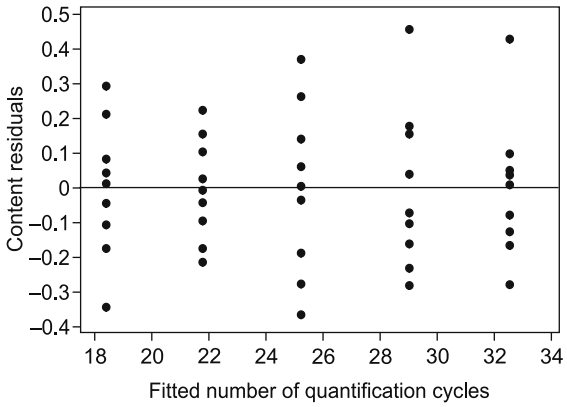

Fig. 6. Content residuals versus fitted *Cq* of 9 replicates (3 replicates of 3 different preparations on 3 different days with a change of sources and analysts) of each of the pork-spiked (100%, 10%, 1%, 0.1% and 0.01%) chicken nuggets.

Residual analysis

Residual analysis facilitates the determination of an amount that is not explained by the *PLS* model of each data point

$$E_i = y_i - \hat{y}_i \tag{5}$$

being

$$\hat{y}_i = b_i x_i \tag{6}$$

where y_i is the actual value and $\hat{y}_i$ is the predicted value of the model; b_i is a regression parameter and x_i is the independent variable [27].

A plot of *Cq* values against the residual content of porcine DNA accommodated all residuals within ± 0.5 of the zero line (Fig. 6), showing a good precision of data for all contents (0.001–10 ng of porcine DNA).

Validation and recovery

A total of 15 model chicken nuggets with 0.01–100% of spiked pork, each in triplicate, were randomly selected and analysed by real-time PCR. The actual and the determined values are shown in Tab. 3. A good recovery, ranging from 96% to 105%, was always determined. Thus, a very low relative error (≤ 5%) was obtained, showing the high accuracy and precision of the developed real-time PCR method.

Analysis of commercial nuggets

We evaluated the suitability of the method for the screening and quantifying pork adulterant in nuggets produced from different meat species. We randomly amplified 10 ng DNA extracted from 27 pork, beef, chicken, mutton and chevon nuggets. Different nuggets were collected on three different days from three different outlets on each day to evaluate the effects of variation in cooking methods as well as the quality and sources of meats species used in each preparation. DNA extracts from all pork nuggets were amplified with a mean *Cq* ranging from 18.15 ± 0.13 to 18.64 ± 0.15 on three different days and analysts with the pork-specific real-time PCR system. ANOVA test revealed no significant difference at $P < 0.05$ with a change of analyst and source, reflecting its suitability to screen commercial nuggets. Analysis of nuggets from other species showed pork contamination in 0, 1, 2 and 1 specimen out of the 27 chicken, beef, mutton and chevon nuggets, respectively. This corresponded to 0%, 3.7%, 7.4% and 3.7% of pork adulteration in the commercial nuggets of the above meat species. Quantitative analysis revealed 20–50% pork in the adulterated nugget formulations. Evaluation of the eco-

nomic impact demonstrated that mixing of pork with beef, mutton and chevon is profitable, but in case of chicken it is not profitable as the price of chicken and pork is almost equal in Malaysia. Amplification of endogenous control lead to *Cq* values ranging from 20.21 ± 0.21 to 20.96 ± 0.24, reflecting the presence of good quality DNA in all samples. While no significant difference was observed in the endogenous *Cq* values of the same species, some differences between the species were observed.

Rojas et al. [18] obtained variable results when quantifying game bird meat species in different commercial meat products. They attributed these to a variation in factors such as meat quality, tissue type, matrix composition and level of processing. We believe these limitations could be effectively addressed if the PCR assay is carefully optimized taking into account all the potential factors of a particular meat product. Consequently, we prepared nuggets from meats on the basis of the tissue composition of a typical nugget meat. We used meats from skeletal muscle, liver, intestine, heart and kidney, carefully estimating the portion of each tissue used in a typical minced meat for nugget preparation. Thus, the numbers of mitochondria and mitochondrial genes, which are tissue-dependent [4], were averaged.

Since the assay was optimized and validated in a mixed background of nugget formulation, we limited the application of the assay to analyze only the commercial nuggets available in Malaysian markets. Although there is a big fluctuation in the processing treatment and parameters of different commercial meat products, there is a little variation of them within the same product, such as nuggets. These variations that may exist between the nugget manufacturing companies, did not affect *Cq* at statistically significant level ($P \leq 0.05$), as reflected by a robust endogenous *Cq* obtained from all commercial nuggets.

The real-time PCR assays developed by Rojas et al. [18] for game bird meat determination were not optimized for a particular commercial meat product. They prepared binary meat mixtures of different percentages. However, they did not consider the effects of typical and complex ingredients found in a particular meat product. In the present assay, we paid attention to all factors, such as tissue composition, typical ingredients and processing parameters, for a typical nugget formulation during the optimization step, and also limited the application of the developed assay to quantify pork only in commercial nuggets. Thus, a high precision and accuracy were a legitimate outcome.

CONCLUSION

A real-time PCR assay suitable for the determination of adulterated pork in nugget formulations of common meat species was developed. The method considered all the potential factors, such as tissue composition, ingredients and processing conditions typically employed in the preparation of a typical commercial nugget while performing model experiment for system optimization. The assay showed 96–105% recovery of pork DNA in fortified chicken nuggets with 0.01–100% of spiked pork, showing a strong agreement with the actual values as well as PLS model-predicted values. The detection limit of the assay was 0.01% of pork in chicken nuggets with a very low relative error (≤ 5%). High precision of all determinations was revealed by residual analysis and sufficient replicate measurements. Analysis of commercial nuggets of common meat species revealed pork adulteration in 3.7–7.4% of commercial nuggets of beef, mutton and chevon, and 0% nuggets of chicken, suggesting that the reason of the substitution was the economic benefit. The high sensitivity, accuracy and precision of the assay suggest that the developed method has a potential to be used by regulatory and enforcement bodies as well as quality control laboratories for Halal and Kosher food verification and certification.

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