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TaqMan probe real-time polymerase chain reaction assay for the quantification of canine DNA in chicken nugget

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\textbf{ABSTRACT}

This paper describes a short-amplicon-based TaqMan probe quantitative real-time PCR (qPCR) assay for the quantitative detection of canine meat in chicken nuggets, which are very popular across the world, including Malaysia. The assay targeted a 100-bp fragment of canine cytb gene using a canine-specific primer and TaqMan probe. Specificity against 10 different animals and plants species demonstrated threshold cycles (Ct) of 16.13 ± 0.12 to 16.25 ± 0.23 for canine DNA and negative results for the others in a 40-cycle reaction. The assay was tested for the quantification of up to 0.01% canine meat in deliberately spiked chicken nuggets with 99.7% PCR efficiency and 0.995 correlation coefficient. The analysis of the actual and qPCR predicted values showed a high recovery rate (from 87% ± 28% to 112% ± 19%) with a linear regression close to unity ($R^2 = 0.999$). Finally, samples of three halal-branded commercial chicken nuggets collected from different Malaysian outlets were screened for canine meat, but no contamination was demonstrated.

\textbf{ARTICLE HISTORY}

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\textbf{KEYWORDS}

qPCR assay; canine species; nugget formulation; halal meat; food adulteration

\section*{Introduction}

Meat is a major source of protein in the human diet with a high demand and high economic value (Soares et al. 2014). Food adulteration with materials derived from meat species of greater or easier availability and/or lower cost is a widespread problem in the meat industry (Drummond et al. 2013). Recent meat adulteration scandals involving horsemeat in Europe (Premanandh 2013) and rat and fox meat in China (D’Amato et al. 2013) have lowered consumers’ trust in the composition of foods products they are purchasing to eat. In addition to allergic reactions and health impacts, consuming ingredients from certain animals, such as porcine, canine and feline species, are forbidden in certain religions, such as Islam, Judaism and Hinduism. Certain animal species are also potential carriers of several zoonotic diseases such as anthrax (bovine, canine and feline species) (Fasanella et al. 2010), human immunodeficiency virus (HIV – chimpanzee species) (Girish et al. 2004), and H5N1 virus (avian species) (Beigel et al. 2005). All these have made it essential to detect and quantify the origin of species in animal-derived materials (Fosse et al. 2008).

Chicken nugget is a type of fast food that is popular in all parts of the world. It can be prepared from deboned meats, vegetable proteins, eggs and a fair portion of dietary fibres (Ali et al. 2012a). However, several meat-forgery scandals such as the horsemeat scandal in Europe involved chicken nuggets (Embiricos 2013). Although canine meat consumption is banned by the Islamic religion and campaigned against by animal right groups (Ali et al. 2014), it has been consumed in many parts of the world including South Korea, China and Vietnam (Podberscek 2009). Since stray dogs are available in many parts of the world, canine meat can be obtained without any offered prices (Totton et al. 2010). Thus, the fraudulent mixing of dog meat in costly meat products such as chicken nuggets cannot be ruled out. In Malaysia, reports have been raised that dog meat is consumed by foreign workers, especially from Myanmar and Vietnam (Nagpal 2008). However, no systematic studies have been published for the detection of dog meat in commercial food products such as in chicken nuggets.

Short-amplicon-length PCR assays are useful in food forensics and other archaeological studies since they
are highly stable under food processing treatments or compromised states (Ali et al. 2012a). Several assays based on conventional PCR have been proposed for the qualitative detection of canine species (Abdulmawjood et al. 2003; Gao et al. 2004; Ilhak & Arslan 2007; Martín et al. 2007; Abdel-Rahman et al. 2009; Ali et al. 2014; Rahman et al. 2014). However, no report has been published for the quantification of canine meats in processed foods. Hence, we describe here a 100-bp amplicon-based quantitative real-time PCR (qPCR) assay for the quantitative determination of canine materials in processed foods such as chicken nugget.

**Materials and methods**

**Collection of samples**

The meat samples of seven commonly used species such as chicken (*Gallus gallus*), turkey (*Meleagris gallopavo*), sheep (*Ovis aries*), goat (*Capra hircus*), beef (*Bos taurus*), buffalo (*Bubalus bubalis*), pig (*Sus scrofa*) and two plant materials such as soybean (*Glycine max*) and wheat (*Triticum aestivum*) which are commonly used in nugget preparation were purchased from supermarkets located in the Kuala Lumpur and Selangor states of Malaysia. Dog (*Canis lupus*) meat from three different animals was collected from Dewan Bandaraya Kuala Lumpur, Malaysia. For commercial chicken nuggets of three different ‘halal’ brands, designated A–C in Table 4, were purchased from different supermarkets and retail outlets in Kuala Lumpur. All samples were collected in triplicate on three different days and transported in an ice-chilled condition (4°C) to prevent the degradation of target analytes. Samples were stored at −20°C for future work and DNA extraction.

**Compliance with ethics requirements**

Ethical clearance (reference number NANOCAT/25/04/3013/MMR (R)) was obtained from the Institutional Animal Care and Use Committee, University of Malaya (UM IACUC). All experiments were conducted and animal meats were handled following national and institutional guidelines.

**Chicken nuggets preparation**

Model chicken nuggets were prepared following Ali et al. (2012a). Briefly, deboned muscle samples of chicken and dog were taken from skeletal muscle (95%), intestine (2.5%), liver (1%), heart (0.5%), kidney (0.5%) and 0.5% skin to simulate the typical animal tissue composition in commercial nuggets. The frozen samples of −20°C were kept overnight at 4°C for thawing and then manually cut into small pieces. For the simulation of dog meat contamination in nuggets, meat mixtures were prepared by spiking 0.0%, 0.01%, 0.1%, 1%, 10% and 100% (w/w) of dog meats in a total volume of 200 g. The meat admixtures were minced twice using a 4 mm plate with meat mincer (Sin Huat Hin, Seremban, Malaysia). To a 200 g portion of each meat mixture, 10 g soybean oil, 10 g textured soya protein, 10 g chilled water, 5 g refined wheat flour, 15 g finely chopped raw carrot, 10 g whole egg liquid, 5 g condiments (onion and garlic paste), 3 g spice mix, 1 g sugar, 2 g sodium chloride, 25 mg sodium nitrite and 0.4 g tetrasodium pyrophosphate were added as according to Ali et al. (2012a). Vigorous blending was performed until an emulsion of each admixture was prepared. The lid of the stainless steel moulds was tightly closed and the emulsions were steam-cooked at an internal temperature of 80–85°C for 15 min. The meat emulsions were then cooled at RT and given a nugget shape.

**Calibration and validation standard**

For calibration set, chicken nuggets were spiked with 0.01%, 0.1%, 1.0% and 10.0% (w/w) of deboned minced meats of dogs. A different set of nuggets was also prepared with a similar composition for the validation of the prediction model following Ali et al. (2012a).

**Extraction of DNA**

DNA was extracted from 25 mg of raw meat samples using a NucleoSpin® Tissue extraction kit (Macherey-Nagel, Duren, Germany) according to the manufacturer’s instructions. From the spiked nugget preparation, DNA was extracted from 1 g sample using CTAB extraction method as described by Ma et al. (2000). It was further purified by using Promega Wizard™ DNA isolation kit (Promega Corporation, Madison, WI, USA). The concentration and purity of the DNAs were determined by using UV-vis spectrophotometer (Libra S70; Biochrom, Cambridge, UK).

**Primer and probe design**

A pair of canine-specific primers (CacytbF and CacytbR) and a 24-nt TaqMan probe (CacytbTqM) targeting a 100-bp fragment of canine cytb gene (Dog: JF489119.1) was designed using publicly available primer3Plus software (see [www.bioinformatics.nl/cgibin/primer3plus/primer3plus.cgi](http://www.bioinformatics.nl/cgibin/primer3plus/primer3plus.cgi)). The 24-nt canine
TaqMan probe (CacytbTqM) was tagged by 6-carboxyfluoresceine (6-FAM) and 3-Iowa black FQ (3-IABkFQ) at the 5′- and 3′-ends. For endogenous control, eukaryotic 18S rRNA-specific primers (Eu18SrRNAF and Eu18SrRNAR) and a TaqMan probe (Eu18SrRNATqM) described by Rojas et al. (2010) were used. All the primers and probe were synthesised and supplied by the 1st BASE Laboratories Sdn Bhd (Selangor, Malaysia) and are included in Table 1.

**Table 1. Primers and probe sequences used in this study.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequences (5´–3´)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CancytbF</td>
<td>CCTTACTAGGAGTATGCTTG</td>
</tr>
<tr>
<td>CancytbR</td>
<td>TGGGTGACTGATGAAAAAG</td>
</tr>
<tr>
<td>CancytbTq</td>
<td>6-FAM/AAGTGGACT/SEN/TGCACTACATCGACACAGCCA</td>
</tr>
<tr>
<td></td>
<td>3IABkFQ</td>
</tr>
<tr>
<td>Eu18SrRNAF</td>
<td>GGTAGTGACGAAAATAACATACAGGCC</td>
</tr>
<tr>
<td>Eu18SrRNAR</td>
<td>ATACGCTATTGGAGCTGGAATTACC</td>
</tr>
<tr>
<td>Eu18SrRNATq</td>
<td>6-FAM/AAGTGGACT/SEN/CATTCCAATTACAGGCCCT/</td>
</tr>
<tr>
<td></td>
<td>3IABkFQ</td>
</tr>
</tbody>
</table>

Real-time PCR assay

Real-time PCR was performed in an Eppendorf Mastercycler ep-realplex machine (Eppendorf, Wesseling, Germany) with a 20 μl reaction mixture consisting of 1× SsoFast probe supermix (Bio-Rad, Hercules, CA, USA), 200 nM of TaqMan probe, 300 nM of each primer and 20 ng of the genomic DNA template. The dilutions were prepared using sterile deionised water. For PCR amplification, a two-step amplification programme was optimised at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 15 s, annealing and extension at 58°C for 20 s. Endogenous control and canine-specific qPCR assays were performed in separate tubes. Each sample and endogenous controls were run in triplicate on three different days by three independent analysts.

Construction of a standard curve and target quantification

The quantification of canine DNA in different chicken nuggets was done by interpolating the value of the quantification cycle (Ct) in a standard curve. The standard curve was generated from Ct values against the known concentration of the DNA. For the calculation of the Ct value of the canine-specific system (CtPS), the following equation was used, as described by Rojas et al. (2010):

\[
CtNPS = CtEP \times CtPS / CtEB
\]

where CtNPS represents the normalised Ct value of the sample with canine-specific PCR system; CtEp is the average Ct value of 20 ng canine DNA from nuggets formulation in endogenous PCR system; and CtEB is the Ct value of the specific nuggets sample with the endogenous PCR system. The validation experiment with chicken nuggets samples at a 95% confidence level with various amount of spiked canine meat did not show any significant changes in the Ct values of the endogenous system. Therefore, it was assumed that CtEp = CtEB and thus the simplified form of the above equation was derived:

\[
CtNPS = CtSP
\]

It demonstrated no significant statistical difference between the normalised Ct and the unprocessed Ct values of the canine-specific system in chicken nugget formulations.

Statistical analysis and validation

Real-time data analyses using an analysis of variance (ANOVA) test were performed by Minitab 14 software (Minitab, State College, PA, USA) and Xlstat software (Addinsosfot, Paris, France). For mean analysis, Tukey’s honest significant difference (HSD) method was used along with Best model selection with adjusted $R^2$. This model can handle a number of variables from ‘min variables’ to ‘max variables’.

Result and discussion

TaqMan real-time PCR assay

This real-time PCR assay for canine DNA quantification targeted a short fragment of the canine cyt b gene (100 bp) using canine-specific primers and a TaqMan probe. For assay normalisation, a 141-bp site of the eukaryotic 18S rRNA gene was used as a reference target (Rojas et al. 2010). The application of the endogenous reference gene in the real-time PCR assay aids in the quantification of accurate target and prevents false-negative detection. It eliminates the effects of other affecting factors such as expired reagents and the presence of nucleic acid inhibitors in reaction vessels (Rojas et al. 2011). For compromised samples such as processed foods, endogenous control is important since degraded and low-purity DNA extracts are often obtained from commercial and processed meat products. An endogenous system may amplify the DNAs from the non-target species and allow the comparison of the signals acquired from the species-specific and endogenous control (Soares et al. 2014). It reduces...
factual errors caused by the standards and the unknown samples (Rojas et al. 2010). For validation of the proposed qPCR assay, we have followed the standard procedure to define the amplification efficiency of the primers and probe (Ali et al. 2012a). Thus, triplicate samples of each amplification target and two sets of canine meat-spiked model nuggets were used to validate this current assay. The potentiality of the proposed assay was verified by successful amplification of the canine-specific target with a high specificity and sensitivity.

For canine specificity, the designed primers and TaqMan probe were tested first in-silico using a blast tool in NCBI (see http://www.ncbi.nlm.nih.gov/blast) and alignment analysis using a ClustalW alignment tool (Thompson et al. 1994) against a total of eight commonly used meat providing animals and two plants species used in nugget formulation. The PCR was then run at a higher annealing temperature (58°C) which prevents non-specific primer binding and cross-species detection (Rahman et al. 2014). For enhanced qPCR signal detection, the intra-molecular distance between the 5′-fluorophore (FAM) and 3′-quencher (Iowa black) was reduced by inserting a second quencher, ZEN probe, at the 10th position of the TaqMan probe. The developed PCR system was tested primarily under pure state and then validated for the analysis of chicken nuggets which contain multiple food ingredients with a complex matrix. A 100-bp target of a multi-copy mitochondrial cyt b gene was selected and short length DNA targets have proven stability and sensitivity even in highly degraded samples and under extreme food processing conditions (Dooley et al. 2004; Rahman et al. 2014).

**Canine specificity**

NCBI blast analysis demonstrated that the primers and probe had 100% identical sequence similarity with the canine cyt b gene. The alignment of both primers and probe sequences using a ClustalW sequence alignment tool showed multiple nucleotide mismatches (forward 4–8, reverse 3–11, probe 5–13) with all the tested meat species (Figure 1(a)). The pair-wise distance of the 100-bp canine site including the primers and TaqMan probe were compared with the retrieved cyt b gene sequences of eight common meat-providing animals and apocytochrome (cob) gene sequences of two plant (stated in the sample collection section) using a maximum composite likelihood method (Tamura et al. 2011). The lowest distance was between dog and sheep (0.26) and the highest was between dog and soybean (0.73). Drawing a 3D plot using mismatched nucleotides in the primers and probe binding site clearly

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**Figure 1.** (colour online) In silico analysis of canine-specific primers and probes along with a 100-bp canine-specific site: (a) position of the primers and probes along with the mismatch count; (b) 3D plot showing a clear discrimination of canine species using oligonucleotide mismatches in the primers and probe binding site; and (c) dendogram built by 100-bp canine-specific site using a maximum composite likelihood method showing the separation of canine species from other common meat-providing animals and common plant species for nugget preparation.
discriminated dog from all other tested species including sheep (Figure 1(b)). Previous studies demonstrates that the presence of a single mismatch in the primer binding site may reduce specificity of the assay and may lead to PCR amplification failure (Smith et al. 2002; Wu et al. 2009). Thus, in-silico analysis reflected the zero probability of primer annealing with any non-target species and cross-species detection. Further, a dendogram with alignment results of the 100-bp canine site with other species well separated the canine species from other meat and plant species commonly used in nugget formulation (Figure 1(c)).

Finally, the cross-specificity of the canine-qPCR system was analysed using 20 ng DNA from the muscle samples of eight meat (dog, chicken, turkey, beef, buffalo, lamb, goat and pig) and two plants species (wheat and soya bean) and an amplification signal was obtained only from the canine target in three repeated PCR runs. The quality of amplifiable DNA in all targets was demonstrated from the amplified endogenous control using universal eukaryotic primers and probe (Rojas et al. 2010). The Ct values using the pure canine DNA target ranged from 16.13 ± 0.12 to 16.25 ± 0.23 and there was no amplification pattern for other animals in a 40-cycle reaction (Table 2). Thus, for raw canine meat using a canine qPCR system the average Ct values of 16.19 ± 0.16 were obtained from nine replicates from three different days. The TaqMan probe qPCR system effectively detected canine-specific targets since both the primers and the probe have a 100% complimentary side and a negative result for other may be due to the presence of a non-complimentary target. The primers showed 100% exclusivity for dogs’ meat as they had 0% false positivity with non-canine samples. The average Ct values for the animals’ and plants’ endogenous qPCR systems were 19.99 ± 1.1 and 31.15 ± 1.5. Although there was a significant different between the species’ DNAs, two major variations in Ct values (11.16) were observed for animals’ and plants’ endogenous control, which may be due to the interspecies’ sequence variation of animals and plants.

Efficiency and LOD

To test the efficiency and LOD of the canine qPCR assay, DNA samples from 100%, 10%, 1%, 0.1% and 0.01% of dog meat-spiked chicken nuggets were analysed. A total of 20 ng DNA from each dog meat-spiked nuggets were used to amplify both the canine target and the eukaryotic control. In real-time PCR assay, DNA quantification is performed from the detected fluorescence signals against the number of cycles on a logarithmic scale where a threshold of detection was slightly above the background noise (Yuan et al. 2006). The number of threshold cycles (Ct) for different percentages (100% to 0.01%) of dog meat contaminated nuggets were ranged from 16.34 ± 0.28 (100%) to 29.23 ± 0.18 (0.01%). Thus, the assay had an LOD of 0.01% for canine meat-spiked nuggets. The mean Ct values of the eukaryotic endogenous control for different canine meat-spiked (0.01–100%) nugget samples were ranged from 19.85 ± 0.57 to 20.20 ± 0.37. The analysis of the endogenous control Ct values at p ≤ 0.05 using an ANOVA test revealed no significant difference of endogenous Ct values for different percentage of canine meat-spiked nuggets. This might be due to the sequence resemblance of 18S rRNA of dog (AY623831.1) and chicken (DQ018752.1), which allowed the similar target site for an absolute quantification even from a different level of dog meat-spiked chicken nuggets. We compared these sequences by alignment and indeed found 100% similarities between chicken and canine species (data not shown).

For the construction of a standard curve using real-time PCR assay, the Ct values obtained from a total of 45 replicates of chicken nuggets (three on three

<table>
<thead>
<tr>
<th>Species</th>
<th>Mean Ct canine-specific PCR system</th>
<th>Positive replicate</th>
<th>Mean Ct endogenous control</th>
<th>Positive replicate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
<td>Day 3</td>
<td>Day 1</td>
</tr>
<tr>
<td>Dog</td>
<td>16.25 ± 0.23</td>
<td>16.20 ± 0.15</td>
<td>16.13 ± 0.12</td>
<td>9/9</td>
</tr>
<tr>
<td>Chicken</td>
<td>40°</td>
<td>40°</td>
<td>40°</td>
<td>0/9</td>
</tr>
<tr>
<td>Turkey</td>
<td>40°</td>
<td>40°</td>
<td>40°</td>
<td>0/9</td>
</tr>
<tr>
<td>Goat</td>
<td>40°</td>
<td>40°</td>
<td>40°</td>
<td>0/9</td>
</tr>
<tr>
<td>Sheep</td>
<td>40°</td>
<td>40°</td>
<td>40°</td>
<td>0/9</td>
</tr>
<tr>
<td>Beef</td>
<td>40°</td>
<td>40°</td>
<td>40°</td>
<td>0/9</td>
</tr>
<tr>
<td>Buffalo</td>
<td>40°</td>
<td>40°</td>
<td>40°</td>
<td>0/9</td>
</tr>
<tr>
<td>Pig</td>
<td>40°</td>
<td>40°</td>
<td>40°</td>
<td>0/9</td>
</tr>
<tr>
<td>Wheat</td>
<td>40°</td>
<td>40°</td>
<td>40°</td>
<td>0/9</td>
</tr>
<tr>
<td>Soybean</td>
<td>40°</td>
<td>40°</td>
<td>40°</td>
<td>0/9</td>
</tr>
</tbody>
</table>

Note: Means with the same letter within the same column are not significantly different at a 5% probability level.
different days for five different samples) with different levels (0.01–100%) of dog meat contamination were analysed at a 95% confidence level. The logarithmic value of canine DNA in each formulation was plotted against the raw Ct values obtained from a different percentage of deliberately spiked samples. Thus, the proposed canine-specific system showed a good linear regression equation with a high correlation coefficient ($R^2 = 0.995$) and slope of $-3.328$ (Figure 2).

For the calculation of the PCR efficiency ($E$) in nugget formulation, the previously described method, $E = \left(10^{(-1/slope)} - 1 \right)$ was used (Fajardo et al. 2008). A PCR efficiency of 99.7% from the present assay was obtained by using the current canine-specific primer and probe. Rodríguez et al. (2005) obtained 68.9% efficiency in raw and an autoclaved pork–beef binary admixture. The LOD of the assay was 0.1% porcine DNA in pork–beef binary mixtures with a longer amplicon (411 bp) target using 12S rRNA porcine-specific gene. Subsequently, Yusop et al. (2012), using a comparatively smaller amplicon (119 bp) target and molecular beacon probe, realised a 96% PCR efficiency. Recently, the extraordinary performance of an mt-cytb-based TaqMan Probe qPCR assay has been reported by Ali et al., (2012b) with 102% PCR efficiency and linear regression of $R^2 = 0.994$, using a 109-bp amplicon target. Hsieh et al. (2001) reported that the mt-cytb gene is more useful for detecting species’ origin in a degraded forensic sample. Thus, the higher efficiency (99.7%) with a high linearity ($R^2 = 0.995$) of the present assay could be conferred to the short-length (100 bp) DNA and multi-copy mitochondrial cyt b gene target.

To obtain the real-time PCR efficiency and LOD, a standard curve can be developed from 10-fold serial dilutions of pure samples (Yusop et al. 2012) or by using DNAs from a binary species background composed of an admixture of two different species (Rodríguez et al. 2005; Rojas et al. 2010). However, ready-to-eat or processed foods are composed of a more complex background with different species, spices and food additives (Tanabe et al. 2007; Rahman et al. 2014). Thus, the development of a standard curve from a pure or binary meat format background has a drawback of accuracy in the quantification of the target species for the commercial or ready-to-eat food products. To overcome this limitation, we prepared typical model commercial nuggets by spiking various percentages of dog meat. For better accuracy, different percentages of deboned meats (95% skeletal muscle, 3% liver, 1% intestine, 0.5% heart and 0.5% kidney) were used in the nugget formulation for the availability of a tissue-dependent mitochondrial genes target (Ali et al. 2012a).

**Assay validation**

For the validation of the developed assay, the actual values (% w/w and ng µl$^{-1}$) in admixed nuggets were compared with qPCR-determined values. A total of 15 model chicken nuggets with 0.01–100% of deliberately contaminated dog meat with chicken were used for assay validation. Very good linearity ($R^2 = 0.999$) was observed while a different percentage of the deliberately canine meat-contaminated nuggets and RT-PCR predicted values were plotted (Figure 3). The present model for the analysis of canine meat-contaminated nuggets showed an excellent recovery rate of $87\% \pm 28\%$ to $112\% \pm 19\%$ (Table 3) for dog meat contamination, where a different percentage of dog meat (% w/w) was admixed with chicken meat for nugget formulation. Furthermore, analysis of the DNA concentration (ng µl$^{-1}$) based on the UV-vis spectrophotometer and of the RT-PCR predicted value also showed a good recovery rate of $89\% \pm 26\%$ to $112\% \pm 19\%$ (Table 3) for the detection of canine DNA (ng µl$^{-1}$).

The qPCR is a well-defined method for determining the prediction value with a higher recovery rate. Ali et al. (2012a) have documented a qPCR assay with a recover rate of 96–105% for pork DNA analysis under nugget formulation. However, to date no assay has been documented for canine species DNA analysis under food matrices. To the best of our knowledge this is first qPCR assay for canine DNA analysis under commercial food matrix such as in nugget formulation.

**Figure 2.** Normalised calibration curve obtained from a different percentage (from 100% to 0.01%) of canine meat-spiked chicken nuggets with Ct values.
Commercial chicken nugget analysis

In this study we tested the potentiality of the qPCR-based assay for commercial sample analysis. To achieve this goal, we run the qPCR assay using 20 ng of DNA extracted from different nugget samples collected from three different outlets across Malaysia on three different days. Evaluation of different commercial chicken nugget samples showed no amplification spectrum for the canine-specific system in 40 cycles of reaction (Table 4). However, the positive control of 0.01% deliberately canine meat-contaminated samples showed a canine-specific amplification pattern at the range of 28.95 ± 0.71 to 29.21 ± 0.61 cycles (Table 4). Amplification of endogenous control from the commercial samples with Ct values of 20.19 ± 0.62 to 21.50 ± 0.48 indicated the presence of good-quality DNA in the samples (Table 4). Thus, the absence of a certain amount of canine DNA in commercial samples was proven by the non-amplification pattern of the DNAs from non-spiked nuggets within 40 cycles of the qPCR reaction.

For the analysis of commercial nuggets, the previously described qPCR assay was for the determination of fraudulent admixing of pork meat (Ali et al. 2012a). The assay was tested on commercial nuggets using the tissue composition for typical meat samples such as skeletal muscle, liver, intestine, heart and kidney to minimise the variation of assay performance. There were no significant differences in endogenous Ct values among the different nuggets (p < 0.05). We have tested this canine-specific assay by optimising the above potential factors and commercial products formulation. No significant difference (p < 0.05) was observed between samples of A versus B and A versus C (Table 4). However, a significant difference (p < 0.05) between B versus C was noticed which

Table 3. Validation of the real-time qPCR assay for determining canine meat/DNA using deliberately contaminated and qPCR-predicted value of canine meat/DNA in chicken nuggets.

<table>
<thead>
<tr>
<th>Canine meat (contamination)</th>
<th>qPCR predicted value</th>
<th>Canine DNA (contamination)</th>
<th>qPCR predicted value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Admixed (%) w/w</td>
<td>Admixed (%) w/w</td>
<td>Recovery w/w (%)</td>
<td>Concentration (ng µl⁻¹)</td>
</tr>
<tr>
<td>100</td>
<td>94.79 ± 8.39</td>
<td>94.56 ± 8</td>
<td>20</td>
</tr>
<tr>
<td>10</td>
<td>11.29 ± 1.95</td>
<td>112 ± 19</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>1.07 ± 0.18</td>
<td>107 ± 18</td>
<td>0.2</td>
</tr>
<tr>
<td>0.10</td>
<td>0.10 ± 0.03</td>
<td>87 ± 28</td>
<td>0.02</td>
</tr>
<tr>
<td>0.01</td>
<td>0.01 ± 0.00</td>
<td>108 ± 21</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Note: Means with the same letter within the same column are not significantly different at a 5% probability level.

Table 4. Commercial chicken nugget analysis using canine-specific qPCR assay.

<table>
<thead>
<tr>
<th>Nugget samples</th>
<th>Mean Ct Canine-specific PCR system</th>
<th>Positive replicate</th>
<th>Mean Ct Endogenous control</th>
<th>Positive replicate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
<td>Day 3</td>
<td>Day 1</td>
</tr>
<tr>
<td>Canine meat spiked</td>
<td>28.95 ± 0.71b</td>
<td>29.21 ± 0.61b</td>
<td>29.36 ± 0.34b</td>
<td>9/9</td>
</tr>
<tr>
<td>A</td>
<td>40b</td>
<td>40b</td>
<td>40b</td>
<td>0/9</td>
</tr>
<tr>
<td>B</td>
<td>40b</td>
<td>40b</td>
<td>40b</td>
<td>0/9</td>
</tr>
<tr>
<td>C</td>
<td>40b</td>
<td>40b</td>
<td>40b</td>
<td>0/9</td>
</tr>
</tbody>
</table>

Note: Means with the same letter within the same column are not significantly different at a 5% probability level.
may be due the variation of the processing treatment or formulations of nuggets from these two commercial brands (Table 4). Rojas et al. (2010) also obtained variable results while quantifying game bird meat species in different commercial meat products. The Malaysian government has prioritised building a world 'halal hub' through the regular surveillance of the food products of local markets with the development of new technology. In line with these objectives, we analysed commercial nugget samples from different outlets of Malaysia and found that no sample was positive for canine meat contamination.

**Conclusions**

A real-time PCR assay to determine canine meat in chicken nugget formulations was developed. The assay considered potential factors such as specificity, processing conditions, ingredients and meat tissue composition to validate the proposed model. A high PCR efficiency of 99.7% and LOD of 0.01% for canine DNA in chicken nuggets were obtained. The model experiment with calibration and validation sets showed no cross-species detection and strong correlation (R² = 0.999) between the actual and predicted values. Finally, analysis of a total of 27 samples of commercial chicken nuggets from Malaysian outlets showed no canine meat contamination. Thus, the assay showed a high potential for laboratory use in canine meat detection.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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