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

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Here we describe a short-amplicon length TaqMan probe quantitative real-time PCR (qPCR) assay for the quantitative detection of the canine meats in chicken nuggets which is 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 analysis against 10 different animals and plants species demonstrated a threshold cycles (C\textsubscript{t}) of 16.13±.12 to 16.25±.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 revealed a high recovery rate (87 ± 28% to 112± 19%) with a linear regression close to unity (R\textsuperscript{2} = 0.999). Finally, samples of 3 halal branded commercial chicken nuggets collected from different Malaysian outlets were screened for canine meat and negative contamination was demonstrated.

\textbf{Keyword:} qPCR assay, Canine meat, Nugget formulation.
Introduction

Meat is a major source of protein in human diet with high demand and 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 wide spread problem in meat industry (Drummond et al. 2012). Recent meat adulteration scandals involving meats from horse in Europe (Reilly 2013), rat and fox meats in China (Kaiman 2013), human meat in the United States (Olumide 2014), have given a great strike on consumers’ trust on the composition of foods we eat. In addition to the allergic reactions and health impacts, ingredients from certain animals such as porcine, canine and feline species are forbidden to consume in certain religious belief such as in Islam, Judaism and Hinduism. Certain animal species are also potential career of several zoonotic diseases such anthrax (canine and feline species) (Anitei 2006), human immunodeficiency virus (chimpanzee species) (Girish et al. 2004), 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 (Corona et al. 2013).

Chicken nugget is a type of fast food that is popular in all parts of the world (Ali, Hashim, Mustafa, Che Man, et al. 2012). It can be prepared from deboned meats, vegetable proteins, eggs and a fair portion of dietary fibers (Ali, Hashim, Mustafa, Che Man, Latif, Islam, Bakar and Rahman 2012). However, several meat-forgery scandals such as horse-meat scandal in Europe involved chicken nuggets (Embiricos 2013). Although canine meat consumptions are banned by Islamic religion and 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 could be procured without any offered prices (Totton et al. 2010). Thus the fraudulent mixing of dog meats in costly meat products such as chicken nuggets cannot be ruled out. In Malaysia, reports have been made that dog meats are consumed by foreign workers, especially from Myanmar and Vietnam origins (Nagpal 2008). However, no systematic studies have been published for the detection of dog meats in commercial food products such as chicken nuggets which is very popular in Malaysia or elsewhere.

Short-amplicon length PCR assays are useful in food forensics and other archeological studies since they are highly stable under food processing treatments or compromised states (Ali, Hashim, Mustafa and CheMan 2012). Several assays based on conventional polymerase chain reaction (PCR) have been proposed for the qualitative
detection of canine species (Abdel-Rahman et al. 2009, Abdulmawjood et al. 2003, Ali, Rahman, Hamid, Mustafa, Bhassu and Hashim 2014, Gao et al. 2004, İlhak and Arslan 2007, Martín et al. 2007, Rahman et al. 2014). However, no report has been published for the quantification of canine meats in processed foods. Hence, we described 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 used in nugget preparation were purchased from the various supermarkets located at Kuala Lumpur and Selangor states of Malaysia. The dog (Canis lupus) meats from three different animals were collected from Dewan Bandaraya Kuala Lumpur, Malaysia. For commercial chicken nuggets of three different “halal brands, designated as A-C in table 4 were purchased from different supermarkets and retail outlets in Kuala Lumpur, Malaysia. All samples were collected in triplicates on three different days and transported at ice chilled condition (4°C) to prevent the degradation of target analytes. Samples were stored at -20°C for future work and DNA extraction.

Chicken nuggets preparation
Model chicken nuggets were prepared following Ali, Hashim, Mustafa, Che Man, Latif, Islam, Bakar and Rahman (2012). Briefly, the 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 samples were chilled overnight at 4 °C, manually cut into small pieces and minced. 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 an adjustable portion of chicken in total volume of 200g. 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. By vigorous blending, an emulsion of each admixture was prepared and steam cooked manually in stainless steel molds. The lid was tightly closed and the emulsions were steam-cooked at an internal temperature of 80–85 °C for 15 min. The meat emulsion was cooled at room temperature and was given to 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 similar composition for the validation of the prediction model following Ali, Hashim, Mustafa, Che Man, Latif, Islam, Bakar and Rahman (2012).

**Extraction of DNA**
DNA was extracted from 25 mg of raw meat samples using NucleoSpin® Tissue extraction kit (Macherey-Nagel, Germany) according to manufacturer’s instructions. For spiked nugget preparations, DNA was extracted from 1 g sample using CTAB method following animal tissue extraction protocol. It was further purified by using Promega Wizard™ DNA isolation kit (Promega Corporation, Madison, USA). The concentration and purity of the DNAs were determined by using UV-vis spectrophotometer (Libra S70, Biochrom, UK).

**Primer and probe design**
A pair of canine specific primers (CacytbF and CacytbR) and a 24-nt TaqMan probe (CacytbTqM) targeting 100-bp fragment of canine cyt b gene (Dog: JF489119.1) was designed using publicly available primer3Plus software (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 18SrRNA-specific primers (Eu18SrRNASF and Eu18SrRNAF) and a TaqMan probe (Eu18SrRNATqM) described by Rojas et al. (2010) were used. All the primers and probes were purchased from IDT, USA and are shown in table1.
**Real-time PCR assay**

Real-time PCR was performed in Eppendorf Mastercycler ep-realplex machine (Eppendorf, Germany) with 20μl reaction mixture consisting of 1× SsoFast probe supermix (Bio-Rad, USA), 200 nM of TaqMan probe, 300 nM of each primers and 20 ng of genomic DNA template. The dilutions were prepared using sterile deionized water. For PCR amplification, a two-step amplification program was optimized 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 triplicates on three different days by three independent analysts.

**Construction of standard curve and target quantification**

The quantification of canine DNA in different chicken nuggets was done by interpolating value of 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 Ct value of the canine-specific system (CtPS), the following equation was used as described by Rojas et al. (2010).

\[ Ct_{NPS} = Ct_{Ep} \times \frac{Ct_{PS}}{Ct_{EB}} \]  \hspace{1cm} (1)

Where \( Ct_{NPS} \) represents the normalized Ct value of the sample with canine-specific PCR system, \( Ct_{Ep} \) is the average Ct value of 20 ng canine DNA from nuggets formulation in endogenous PCR system, and \( Ct_{EB} \) is the Ct value of the specific nuggets sample with the endogenous PCR system. The validation experiment with chicken nuggets samples at 95% confidence level with various amount of spiked canine meat did not show any significant changes in the Ct values of endogenous system. Therefore it was assumed that \( Ct_{EP} = Ct_{EB} \) and thus the simplified form of the above equation 2 was derived.

\[ Ct_{NPS} = Ct_{SP} \]  \hspace{1cm} (2)

It demonstrated no significant statistical difference between the normalized Ct and the unprocessed Ct of the canine-specific system in chicken nuggets formulations.
Statistical analysis and validation
Real time data analyses using ANOVA test were performed by Minitab 14 software (Minitab, State College, Pennsylvania, USA) and XLstat 2013 (Addinosfot, France). For mean analysis, Tukey’s Honesty Significant Difference (HSD) method was used along with Best model selection with Adjusted R². This model can handle a number of variables varying 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 canine cytb gene (100 bp) using canine specific primers and TaqMan probe. For assay normalization, a 141 bp site of eukaryotic 18S rRNA gene was used as reference target (Rojas et al. 2010). The application of the endogenous reference gene in real time PCR assay aids in the quantification of accurate target and prevent false negative detection. It eliminates the effects of other affecting factors such as expired reagents and presence of nucleic acid inhibitors in reaction vessels (Rojas et al. 2011). For compromised, samples such as processed foods, endogenous control is important since disintegrated and low purity DNA extracts are often obtained from commercial and processed meat products. An endogenous system traces unamplified DNA by the species-specific detectors and supports in comparison of signals acquired from the species-specific and endogenous control (Soares et al. 2013; Soares et al. 2014). It reduces factual errors caused by the standards and the unknown samples (Rojas et al. 2010). For the validation of proposed qPCR assay, we have followed the standard analytical procedure to define the amplification efficiency of the primers and probe. 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 blast tool in NCBI and alignment analysis using ClustalW alignment tool against a total of 8 commonly used meat providing animals and 2 plants species used in nugget formulation. Then the PCR was run at higher annealing temperature (58⁰ C) which prevents
non-specific primer binding and cross-species detection (Rahman, Ali, Hamid, Mustafa, Hashim and Hanapi 2014). To amplify PCR signals, 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 multi-copy mitochondrial cytb gene was selected and short-length DNA targets have proven stability and sensitivity even in highly degraded samples and under extreme food processing conditions (Ali, Hashim, Mustafa and CheMan 2012, Ali, Rahman, Hamid, Mustafa, Bhassu and Hashim 2014).

**Canine specificity**

NCBI blast analysis demonstrated the primers and probe had 100% identical sequence similarity with canine cytb gene. Alignment of both primers and probe sequences using ClustalW sequence alignment tool showed multiple nucleotide mismatches (forward 4-8, reverse 3-11, probe 5-13) with all the tested meat species (Figure 1a, Figure 1c,). The pairwise distance of the 100bp canine site including the primers and Taqman probe were compared with the retrieved cytb gene sequences of 8 common meat-providing animals and apocytochrome (cob) gene sequences of 2 plant (stated in sample collection section) using 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 discriminated dog from all other tested species including sheep (Figure 1a). Previous studies demonstrates that the presence of 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 b).

Finally, the cross-specificity of the canine-qPCR system was analyzed using 20 ng DNA from the muscle samples of 8 meat (dog, chicken, turkey, beef, buffalo, lamb, goat and pig) and 2 plants species (wheat and soya bean) and amplification signal was obtained only from canine target in three repeated PCR run. The eminence 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 were ranged from 16.13±.12 to 16.25±.23 and there were no amplification pattern for other animals in 40-cycle reaction (Table 2). Thus for raw canine meat using canine qPCR system the average Ct values of 16.19±.16 were obtained from 9 replicates from 3 different days. The TaqMan probe qPCR system effectively detected canine specific targets since both the primers and probe have 100% complimentary side and negative result for other may due to the presence of non complimentary target. The average Ct values of the animals and plants endogenous qPCR systems were 19.99±1.1 and 31.15±1.5. Although there was significant different between the species DNAs but two major variations of 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 detection limit**

To test the efficiency and detection limit of the canine qPCR assay, DNA samples from 100%, 10%, 1%, 0.1% and 0.01% of dog meat spiked chicken nuggets were analysed. Twenty ng DNA from each dog meat spiked nuggets were used to amplify both the canine target and 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 (Ali, Hashim, Mustafa, Che Man, Latif, Islam, Bakar and Rahman 2012). The number of threshold cycles (Ct) at which the fluorescence exceeded the threshold for different percentages (100% - 0.01%) of dog meat spiked nuggets were between 16.34±0.28 (100%) and 29.23±0.18 (0.01%) (data not shown). Thus the assay had the detection limit 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 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 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 different days for five different samples) with different level (0.01% – 100%) of dog meat contamination were analyzed at 95% confidence level. The logarithmic value of canine DNA in each formulation was plotted against the raw Ct values obtained from 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, $E = \left[10^{(-1/\text{slope})} - 1\right]$ 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 autoclaved pork–beef binary admixture. The detection limit 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 realized 96% PCR efficiency. Recently, Ali, Hashim, Mustafa, Che Man, Latif, Islam, Bakar and Rahman (2012) using Taqman Probe based qPCR assay reported an efficiency of 93.8% for complex form of meat adulteration using a short 109 bp amplicon target of cytb gene. 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 cytb gene target.

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

**Assay validation**

For the validation of the developed assay, the actual values in admixed nuggets were compared with qPCR-determined values. A total of 15 model chicken nuggets with 0.01% – 100% of spiked dog meat were used for assay validation. A very good linearity (R² = 0.999) was observed when different percentage of the deliberately contaminated canine meat nuggets and RT-PCR predicted values were plotted (Figure 3). Thus, the present model for analysis of canine meat contaminated nuggets (%, w/w) showed an excellent recovery rate of 87±28% to 112±19% for dog meat contamination (Table 3). Furthermore, analysis of the DNA concentration (ng/µl) based on the UV-vis spectrophotometer and of the RT-PCR predicted value also showed a good recovery rate of 89 ± 26% to 112±19% for the detection of canine DNA (Table 3).

The qPCR is a well defined method for determining the prediction value with a higher recovery rate. Ali, Hashim, Mustafa, Che Man, Latif, Islam, Bakar and Rahman (2012) have documented a qPCR assay with a recover rate of 96% to 105% for pork DNA analysis under nugget formulation. However, up to date, no assay was documented for canine species DNA analysis under food matrices. To the best of our knowledge this is first validated qPCR assay for canine DNA analysis under commercial food matrix such as in nugget formulation.

**Commercial chicken nugget analysis**

In this study we tested the potentiality of the qPCR based assay for commercial sample analysis. For achieving 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 canine specific amplification pattern at the range of 28.95±.71 to 29.21±.61 cycles (Table 4). Amplification of endogenous control from the commercial samples with Ct values of 20.19±.62 to 21.50±.48 indicated the presence of good quality DNA in the samples (Table 4). Thus, the absence of the canine DNA in commercial samples was proven by the non-
amplification pattern of the DNAs from non-spiked nuggets within 40 cycles of qPCR reaction.

For analysis of commercial nugget, previously described qPCR assay was for determination of fraudulent admixing of pork meat (Ali, Hashim, Mustafa, Che Man, Latif, Islam, Bakar and Rahman 2012). The assay was tested under commercial nuggets using the tissue composition for typical meat samples such as skeletal muscle, liver, intestine, heart and kidney to minimize the variation of the assay performance. There were no significant differences in endogenous Ct value among the different nuggets (P <0.05). We have tested this canine specific assay with optimizing the above potential factors and commercial products formulation. No significant difference (P <0.05) was observed between samples of A vs B and A vs C (Table 4). However, a significant difference (P <0.05) between B vs C have been noticed which 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 been prioritizing the build-up of world “Halal hub” through the regular surveillance of the local markets along with the development of technological tools. Thus the detection of canine non-contaminated nuggets was not a surprise in Malaysian perspectives.

Conclusion
A real-time PCR assay to determine canine meats in chicken nugget formulations was developed. The assay considered all the 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 detection limit 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 ($R^2 = 0.999$) between the actual and predicted values. Finally, analysis of total 27 samples of commercial chicken nuggets from Malaysian outlets revealed no canine meat contamination. Thus the assay showed its high potentiality for laboratory use in canine meat detection.
Acknowledgements
This research was supported by University of Malaya research grant no: RU002-2014 of Prof. SBA Hamid, GC001C-14SBS of Prof. WJ Basirun and GC001A-14SBS of Dr. ME Ali.

This research was jointly supported by University of Malaya research grant no. GC001A-14SBS of Dr. ME. Ali and grant no: RU002-2014 of Prof. SBA. Hamid. M.M. Rahman is thankful to Prof. WJ Basirun for providing salary support through the grant no GC001C-14SBS.

Conflict of Interest
The authors do not have any conflict of interest to publish this manuscript.

Md. Mahfujur Rahman declares that he performed this work under the supervision of Md. Eaqub Ali and he has no conflict of interest to publish this paper.
Sharifah Bee Abd Hamid declares that she supported Md. Mahfujur Rahman from her research grant to perform this work and she has no conflict of interest to publish this paper.
Wan Jefrey Basirun declares he is providing salary to Md. Mahfujur Rahman from his research grant and he has no conflict of interest to publish this paper.
Subha Bhassu declares she helped Md. Mahfujur Rahman for real time probe designing and biomarker specificity analysis and she has no conflict of interest to publish this paper.
Shuhaimi Mustafa declares that he ensured lab facilities for this work and he has no conflict of interest to publish this paper.
Nur Raifana Abdul Rashid declares that he helped in collection of sample and preparation of experimental samples and she has no conflict of interest to publish this paper.
Mohd Nasir Mohd Desa declares that he helped Md. Mahfujur Rahman in real time data analysis and he has no conflict of interest to publish this paper.
Md. Eaqub Ali declares that he received funding support from the University of Malaya, supervised this work and edited manuscript and he has no conflict of interest to publish this paper.
Compliance with Ethics Requirements
Ethical clearance of ref. no: NANOCAT /25/04/3013/ MMR (R) was obtained from the Institutional Animal Care and Use Committee, University of Malaya (UM IACUC), and all experiments were conducted and animal meats were handled following the national and institutional guideline.
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domestica) meats by PCR analysis targeting the mitochondrial D-loop and the nuclear melanocortin receptor 1 (MC1R) genes. Meat Sci.78:314-322.


Captions

Figure Captions

Figure 1. Insilco analysis of canine specific primers and probes along with 100 bp canine specific site. Shown are in (a) the position of the primers and probes along with the mismatch count; in (b) 3D plot showing a clear discrimination of canine species using oligonucleotide mismatches in the primers and probe binding site and in (c) dendogram built by 100 bp canine specific site showing the separation of canine species from other common meat providing animal and plant species.

Figure 2. Normalized calibration curve obtained from different percentage (100% - 0.01%) of canine meat spiked chicken nuggets with Ct values.

Figure 3. Relationship between actual and RT-PCR predicted value of 0.01 to 100 % (w/w) canine meat admixed chicken nugget. Inset is the relationship between actual and RT-PCR predicted value of 0.01 to 1 % (w/w) canine meat admixed chicken nugget.

Tables Captions

Table 1. Primers and probe sequences used for this study

Table 2. Number of fluorescent quantification cycles (Ct value) from different species DNA.

Table 3. Validation of the real time PCR for canine species detection

Table 4. Commercial nugget sample analysis
Figures

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\[ y = -3.3283x + 22.716 \]

\[ R^2 = 0.995 \]
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<thead>
<tr>
<th>Name</th>
<th>Sequences (5’ - 3’)</th>
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<td>Eu18SrRNATq</td>
<td>6-FAM/AAGTGGACT/ZEN/CATTCCAATTACAGGCCCT/3IABkFQ</td>
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<table>
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<th>Mean Ct Canine specific PCR system</th>
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<th>Mean Ct Endogenous control</th>
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<td>Day2</td>
<td>Day3</td>
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<td>16.20±.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.13±.12&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Goat</td>
<td>40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0/9</td>
</tr>
<tr>
<td>Sheep</td>
<td>40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0/9</td>
</tr>
<tr>
<td>Beef</td>
<td>40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0/9</td>
</tr>
<tr>
<td>Buffalo</td>
<td>40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0/9</td>
</tr>
<tr>
<td>Pig</td>
<td>40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0/9</td>
</tr>
<tr>
<td>Wheat</td>
<td>40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0/9</td>
</tr>
<tr>
<td>Soybean</td>
<td>40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0/9</td>
</tr>
</tbody>
</table>

Means with the same letter within the same column are not significantly different at 5% probability level.
Table 3. Validation of the real time PCR assay for canine species detection

<table>
<thead>
<tr>
<th>Admixed, %, w/w</th>
<th>Concentration, ng/µl</th>
<th>RT-PCR predicted canine meat contamination</th>
<th>Admixed, %, w/w</th>
<th>Recovery, w/w (%)</th>
<th>Concentration, ng/µl</th>
<th>Recovery, ng/µl (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>20</td>
<td>94.79±8.39</td>
<td>94.56±8</td>
<td>19±1.712</td>
<td>95±9</td>
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</tr>
<tr>
<td>10</td>
<td>2</td>
<td>11.29±1.95</td>
<td>112±19</td>
<td>2.24±0.389</td>
<td>112±19</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.2</td>
<td>1.07±0.18</td>
<td>107±18</td>
<td>0.20±0.028</td>
<td>101±14</td>
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<tr>
<td>0.10</td>
<td>0.02</td>
<td>0.10±0.03</td>
<td>87±28</td>
<td>0.02±0.005</td>
<td>89±26</td>
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<tr>
<td>0.01</td>
<td>0.002</td>
<td>0.01±0.00</td>
<td>108±21</td>
<td>0.002±0.0004</td>
<td>108±21</td>
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</tbody>
</table>
Table 4. Commercial nugget sample analysis

<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>Day1</td>
<td>Day2</td>
<td>Day3</td>
<td></td>
</tr>
<tr>
<td>Canine meat spiked</td>
<td>28.95±.71a</td>
<td>29.21±.61a</td>
<td>29.36±.34a</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>

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