Polymerase chain reaction assay targeting cytochrome b gene for the detection of dog meat adulteration in meatball formulation

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A B S T R A C T

A polymerase chain reaction (PCR) assay for the assessment of dog meat adulteration in meatballs was developed. The assay selectively amplified a 100-bp region of canine mitochondrial cytochrome b gene from pure, raw, processed and mixed backgrounds. The specificity of the assay was tested against 11 animals and 3 plants species, commonly available for meatball formulation. The stability of the assay was proven under extensively autoclaving conditions that breakdown target DNA. A blind test from ready to eat chicken and beef meatballs showed that the assay can repeatedly detect 0.2% canine meat tissues under complex matrices using 0.04 ng of dog DNA extracted from differentially treated meatballs. The simplicity, stability and sensitivity of the assay suggested that it could be used in halal food industry for the authentication of canine derivatives in processed foods.

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1. Introduction

The prospects for Halal meats and meats products are rapidly expanding (Ali, Hashim, Dhahi, Mustafa, Man, et al., 2012). Currently, halal food consuming population has reached to 1.8 billion and the turnover of halal market has exceeded USD 661 billion (Ali, Kashif, et al., 2012). Several factors including increasing workloads are pushing potential halal consumers to spend more time in workplaces, leaving no time for self-cooking. Thus a growing number of people are increasingly being forced to eat readymade foods such as burger, pizza, hot dogs, meatball, soups and so on (Ali, Kashif, et al., 2012). Due to the specialized preparation of halal meats, the prices for halal brands are higher, especially in those countries where Muslims are the minority. Thus the fraudulent labelling of “halal” brands is prevalent (Ali, Hashim, Mustafa & Che Man, 2012). To cope up with the situation demands and business requirements, many countries including Malaysia, Indonesia, Thailand, Singapore, China, Brazil, Australia and New Zealand are having regulatory bodies to protect the sanctity of Halal food markets (Ali, Kashif, et al., 2012). Thus the food manufacturers, marketers and regulators need innovative, easily performable and improved authentication techniques for the verification of halal brands.

Meatballs made up with comminute meats are very popular throughout the world including Malaysia, Indonesia, China, Vietnam, India, the USA and the Europe (Ali, Hashim, Mustafa, Che Man, Dhahi, et al., 2012; Rohman, Sismindari, Erwanto, & Che Man, 2011). Dog meat is a potential adulterant in halal foods since stray dogs are available in many countries without any offered prices. Reports have been made for the consumption of dog meats in certain countries such as Vietnam, South Korea and China (Bartlett & Clifton, 2003; Podberseck, 2009). Foreign workers, especially from Myanmar and Vietnam origins, are reported to consume stray dog meat in Malaysia. However, no survey is made to verify the mixing of dog meats in commercial meat products across Malaysia or elsewhere in the world. The mixing of dog origin materials in food is a serious issue in many religions including Islam and Buddhism (Khattak et al., 2011; Mahanarongchai & Marranca, 2007).

Several methods such as SYBR green real-time PCR (Farrokhi & Jafari Joozani, 2011), molecular beacon real-time PCR (Yusop, Mustafa, Che Man, Omar, & Mokhtar, 2012), TaqMan probe real-time PCR (Ali, Hashim, Dhahi, et al., 2012), electronic nose coupled with gas chromatography–mass spectrometry (Nurjuliana, Che Man, Mat Hashim, & Mohamed, 2011), Fourier transform infrared spectroscopy (Rohman et al., 2011), enzyme-linked immunosorbant assay (Asensio, González, García, & Martin, 2008), PCR-RFLP (Ali, Hashim, Mustafa, & Che Man, 2012) and nanoparticle sensors coupled with optical or fluorescence spectroscopy (Ali et al., 2011) have been proposed for the authentication of meat species. Although cumbersome to some extent, the conventional species specific PCR assay is an easily affordable and reliable method for the routine analysis of animal meat products in food industry (Arslan, Ilhak, & Calicioglu, 2006; Matsunaga et al., 1999). For dog...
meat detection, a total of five PCR based assays have been documented (Abdel-Rahman, El-Saadani, Ashry, & Haggag, 2009; Abdulmawjood, Schönenbrücher, & Bülte, 2003; Gao, Xu, Liang, Zhang, & Zhu, 2004; Illhak & Arslan, 2007; Martin et al., 2007). However, most of them were of longer amplicon length (> 150 bp) and none of them was tested under commercial matrices.

In this paper, we have described a short amplicon length conventional PCR assay targeting 100 bp region of cytb gene for the detection of canine tissues in ready to eat chicken and beef meatballs. We tested the assay under various backgrounds and processing conditions and high stability and sensitivity were obtained.

2. Materials and methods

2.1. Sample collections

Meat samples of 9 commonly available animals (chicken, duck, turkey, quail, goat, sheep, beef, buffalo and pig) and 3 plants species (onion, garlic and tomato) were purchased in triplicates from various supermarkets located at Serdang, Petaling Jaya and Kuala Lumpur in Malaysia. The typical fresh dog and cat meats were collected from euthanized stray dogs and cats from Jabatan Kesihatan Dewan Bandaraya Kuala Lumpur (DBKL), Malaysia. The post-mortem dog meat samples were also collected from the Faculty of Veterinary Science in University Putra, Malaysia. Commercial meatballs of five different halal brands were purchased from Serdang, Petaling Jaya and Kuala Lumpur in Malaysia in triplicates on three different days. All the collected samples were transported under ice chilled condition (4 °C) and were stored at −20 °C for further processing and DNA extraction.

2.2. Ternary admixture preparation

To evaluate the performance of the PCR assay for canine meat detection in processed food, ternary admixtures composed of different percentages of dog meat were prepared according to Ali, Hashim, Mustafa, and Che Man (2012). Briefly, to obtain 1%, 0.5%, 0.2%, 0.1%, and 0.01% of dog meat admixtures, dog meat was mixed with chicken meat and wheat flour in the ratio of 2:98:100; 1:99:100, 0:4:99:6:100, 0.2:99:8:100 and 0.02:99:98:100. Finally, hundred millilitres (100 ml) of deionized water was added to the admixtures and vigorously ground with a blender to obtain a homogenous semi solid-slurry. All admixtures were prepared on three different days by three independent analysts and autoclaved at 120 °C under 45-psi pressure for 2.5 h. Thus prepared samples were kept at −20 °C for further DNA extraction.

2.3. Meatball preparation

Pure meatballs were prepared according to Rohman et al. (2011) with balanced amount of grinded chicken, beef and dog meat with cooking salt, garlic and other ingredients as shown in Table 1. To obtain dog meat contaminated meatballs 1%, 0.5%, 0.2%, 0.1%, and 0.01% of dog meat were added with 100 g of chicken and beef meat in the formulation. The meats with all other ingredients were mixed well by vigorous blending and the emulsified homogenous meat mixtures were mechanically given into ball shape. To simulate cooking and extensive autoclaving effect thus prepared raw meatballs were subjected to cooking at 100 °C for 90 min and autoclaved at 120 °C under 45-psi pressure for 2.5 h. All samples were prepared on three different days by three independent analysts and were stored at −20 °C for DNA extraction.

2.4. DNA extraction

DNA was extracted from 25 mg of raw and treated meat samples using NucleoSpin® Tissue DNA extraction kit (Macherey-Nagel, Germany) following manufacturer’s instructions. DNA was extracted from plants, admixed and commercial samples from 100 mg specimen using the CTAB method and subsequent purification was performed using Promega Wizard™ DNA isolation kit (Promega Corporation, Madison, USA). Extracted DNA was analyzed by gel image capturing after running the total DNA in 1% agarose gel containing 1 μg/ml ethidium bromide in 0.5% Tris Borate buffer (TBE) for 45 min at 100 volt. The concentration and purity of DNA were determined using a spectrophotometer (Biochrom Libra S80—Cambridge, England).

2.5. Canine specific primer design

The hyper variable region of the dog cytb gene (Dog: JF489119.1) was identified through alignment analysis with the cytb genes of 8 common halal meat species (Chicken: EU839454.1, Turkey: HQ122602.1, Duck: HQ122601.1, Quail: EU839461.1 Beef: EU807948.1, Buffalo: D32193, Sheep: EU365990.1, Goat: EU130780.1, 2 non halal meat species (Pig: GIU135837.1, Cat: AB194817.1) and apocytochrome b (cob) gene of 3 plant species (Tomato: XM004251454.1, Garlic: AF356823.1, Onion: GIU253040.1) using mega 5 software (Tamura et al., 2011) and clustalW alignment tool (Thompson, Higgins, & Gibson, 1994). Thus found hyper variable regions were used to design a pair of canine specific primers (Forward 5′ CTCCTACTAGGCATGCTGG 3′ and Reverse: 5′ TGGGTGTACTGATGAAAAC 3′) using primer3plus software (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The canine specificity of the designed primers was theoretically confirmed through “BLAST” analysis in NCBI data base (http://www.ncbi.nlm.nih.gov/blast). Primers were purchased from the 1st BASE Laboratories Pte Ltd (Selangor, Malaysia).

2.6. PCR assay optimization

PCR assay was run in a gradient thermocycler (Eppendorf, Germany), using 20 μl of reaction mixture composed of 1x PCR master mix (Promega, Promega Corporation, Madison, USA) containing 50 units/ml of Taq DNA polymerase (supplied in a proprietary reaction buffer pH 8, 400 μM each dATP, dGTP, dCTP, dTTP and 3 mM MgCl2), 100 nM of each primer and 20 ng of total DNA. PCR cycling was done using an initial denaturation at 94 °C for 3 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 1 min. The final extension was performed at 72 °C for 5 min. The separation of PCR products was performed in 1% agarose gel (Promega, Madison, USA) in 1x LB buffer of pH 8.0 at a constant voltage of 170 V for 15–20 min, pre-stained with 6x loading dye and using a 100 bp DNA ladder (Fermentas, USA) as reference standard. PCR product was visualized on ethidium bromide stained agarose gel using a gel image documentation system (Alphalmager HP; California, USA).

2.7. Pair wise distance and phylogenetic tree

For pair wise distance and phylogenetic analysis, sequencing results obtained from 100 bp PCR product were aligned with the retrieved cytb/
cob gene sequences of the potential species using ClustalW sequence alignment tool (Thompson et al., 1994). To study pair wise distance between dog and other aligned species, the consensus regions of the gene were used and a phylogenetic tree was built using molecular evolutionary and phylogenetic analysis software, MEGA version 5 (Tamura et al., 2011).

2.8. Canine specificity

The canine specificity of the assay was tested in three steps. In step 1, the retrieved DNA sequences were aligned along with the primer sequences using mega 5 (Tamura et al., 2011) software and clustalW alignment tool for mismatch detection. A 3D plot was built using the number of mismatches and pairwise distance data to find the discriminating properties and molecular orbit of the primers using XLSTAT version 2013.1.01 (Addinsoft, 2013). In step 2, cross species testing was performed in a real PCR run using DNA extracted from the potential meat and plant species for meatball preparation. Finally, in step three, PCR product was purified using Qiaquick PCR purification kit (Qiagen, USA) and sequencing result was analyzed using the BLAST local alignment tool in NCBI data base to confirm the canine cytb gene specificity. The sequencing was done by the 1st Base Laboratories Pte. Ltd., Selangor, Malaysia.

3. Result and discussion

3.1. DNA isolation and PCR optimization

DNA was extracted from raw meat samples using the spin column based extraction technique which is known to reduce DNA loss during aqueous and organic phase separation (Karabasanavar et al., 2011). The realized DNA yield was 180–230 ng per milligram of meat sample and the purity of all DNA samples was 1.90–2.0 (A260/A280). Higher annealing temperature increases primer specificity and reduces nonspecific PCR amplification (Ali, Hashim, Mustafa, & Che Man, 2012; Wu, Hong, & Liu, 2009). Hence, through a repeated run of gradient PCR (data not shown), an optimized higher annealing temperature (58 °C) was used for all PCR run in this study.

3.2. Pair wise distance and phylogenetic tree

Pair wise distance and phylogenetic tree was constructed using alignment analysis of a 100 bp region of the cytb gene obtained from the sequencing of the PCR products realized under various conditions. The consensus gene sequences including primer binding sites were aligned with a total of 11 animal and 3 plant species. Pair wise distance obtained by the maximum composite likelihood method (Tamura, Nei, & Kumar, 2004; Tamura et al., 2011) showed the highest distance between dog and garlic (1.992) and close relationship of dog with sheep (0.256) and similar result was noticed after construction of a phylogenetic tree using the neighbor-joining method (Saitou & Nei, 1987) (Fig. 1). However, the probability of non specific amplification of closely related sheep DNA was eliminated due to the presence of multiple (> 5 nt) mismatches in the reverse and forward primer binding sites (Ali, Hashim, Mustafa, & Che Man, 2012).

3.3. Canine specificity under pure states

The designed primers were aligned against cytb/cob gene sequences of total 14 species including 9 halal, 2 non-halal and 3 plants species to find the distinct specificity of the primers at theoretical level. A 3D plot using the number of mismatches in the primer binding sites and pair wise distances of 100 bp canine specific target site showed a clear discrimination of the dog species from all other potential species for meatball preparation (Fig. 2). The primer pairs were also checked against other animal and plant species using BLAST search in NCBI data base. Complete identity was found only with the canine cytb gene. In real PCR run, the assay amplified only 100 bp canine cytb gene target (Fig. 3). Sequencing of PCR product also confirmed the specificity for the canine cytb gene.

It has been quoted in several reports that the efficiency of the PCR assay might reduce or end up with PCR amplification failure because of the presence of single mismatches in the primer binding region (Ali, Hashim, Mustafa, & Che Man, 2012; Smith, Vigilant, & Morin,
its high sensitivity and specificity of chicken and wheat contaminations of dog meat. The assay successfully detected canine specimens even under complex background. The percentage mismatch (≥5 nucleotide) in the primer binding sites with other 13 potential species for meatball preparation and in real PCR run 100% canine specificity was confirmed. Previously, different PCR assays using mitochondrial whole genome (322 bp) (Ilihak & Arslan, 2007), cytb (808 bp) (Abdel-Rahman et al., 2009; Abdulmawjood et al., 2003), D-loop (213 bp) (Gao et al., 2004) and 12S rRNA (101 bp) (Martin et al., 2007) have been proposed for dog meat detection. However, four of these assays used longer sized amplicon (≥213 bp) (Abdel-Rahman et al., 2009; Abdulmawjood et al., 2003; Gao et al., 2004; Ilihak & Arslan, 2007), which may breakdown under extensive autoclaving and harsh processing conditions (Ali et al., 2011; Rojas et al., 2010). Recently, short amplicon-length PCR assays are appreciated for the authentication of meat-species in processed foods (<150 bp) (Ali, Hashim, Mustafa, & Che Man, 2012; Rojas et al., 2010). Martin et al. (2007) proposed a PCR assay targeting 101 bp region of the canine 12S rRNA gene which should be suitable for the analysis of processed foods. However, the latest cytb-based species identification scheme showed a more accurate reconstruction of mammalian phylogeny with higher resolution (Nicolas et al., 2012). This is probably because of the higher rate of intra-species conservation and protection of the cytb gene over its counterparts. Hence, we speculated that mt-cytb based short amplicon length (100 bp) PCR assay would give better detection of canine derived materials from raw as well highly processed meatballs.

3.4. Specificity and sensitivity in ternary meat mixture

Mt-cytb based canine specific PCR assay developed here was subsequently tested for the specificity and sensitivity under ternary admixed background composed of chicken meat and wheat flour contaminated with 1% to 0.01% of dog meat. The assay successfully detected canine specific target as low as 0.1% of dog meat (0.02 ng dog DNA) from contaminated admixtures of chicken and wheat flour (Fig. 4), reflecting its high sensitivity and specificity even under complex background.

The sensitivity of the dog specific PCR assay documented by Martin et al. (2007) for a 101 bp fragment of the 12S rRNA gene in meat-oat binary admixtures under normal autoclaving condition was 0.1% (0.125 ng). Compared to this assay, our assay was 6.25 times more sensitive in terms of the amount of template DNA used (125 ng vs. 20 ng) and in terms of mixed meat matrices (ternary meat mixtures) under extensive autoclaving. To the best of our knowledge, for the detection of dog meat the highest detection limit (0.01%) was reported by Abdulmawjood et al. (2003) in a PCR-RFLP assay targeting a 808 bp fragment of the mt-cytb gene. However, Abdulmawjood et al. (2003) did not mention how much DNA template they used. The percentage composition, which is an undefined method for concentration expression, can be manipulated through the use of higher amount of template DNA. Furthermore as illustrated earlier for extensive process food analysis, certain literatures reported for the better sensitivity and stability of the short amplicon based PCR assays over the longer ones (Ali, Hashim, Mustafa, & Che Man, 2012; Rojas et al., 2010). Hence, the sensitivity of the PCR-RFLP assay documented by Abdulmawjood et al. (2003) cannot be higher than the present one and Martin et al. (2007).

3.5. Specificity and sensitivity under meatball matrices

Mince meat are often added in commercial food products (Tanabe et al., 2009) and meat adulteration often takes place under mixed and processed conditions (Ali, Hashim, Mustafa, & Che Man, 2012). Therefore, we tested the performance of the assay under complex background of meatballs under pure and different percentages of dog meat admixed condition with chicken and beef meatballs (1% to 0.01%). Thus prepared meatballs were further boiled at 100 °C for 90 min and autoclaved at 120 °C at 45 psi for 2.5 h to simulate cooking and extensive autoclaving. Clear 100 bp PCR products were observed from pure dog meatballs (data not shown) and as low as 0.2% of dog meat contaminated chicken and beef meatballs (Fig. 5). Thus 0.2% dog meat contaminated meatballs
were used as a positive control for screening the halal logo containing commercial chicken and beef meatballs obtained from various supermarkets across Malaysia. In a blind experiment, five commercial brands (A–E) of chicken and beef meatballs were screened in triplicates on three different days against the 0.2% positive control. While all positive controls amplified the selective PCR products specific for canine cytb gene, no commercial samples were found to be positive (Table 2 and Fig. 6).

Although five different PCR assays were previously proposed for canine meat detection (Abdel-Rahman et al., 2009; Abdulmawjood et al., 2003; Gao et al., 2004; Ilhak & Arslan, 2007; Martin et al., 2007) none of them were tested for any commercial meat products. Therefore, we analysed our assay performance in pure and contaminated state of commercial meatballs. The presence of various additives and inhibitors in commercial meat and food products might prevent the primer binding at specific sites and reduce the amplification efficiency, diminishing the sensitivity and specificity of a PCR assay (Bottero, Civera, Anastasio, Turi, & Rosati, 2002; Calvo, Zaragoza, & Osta, 2001; Di Pinto, Forte, Conversano, & Tantillo, 2005). Hence, a constant detection limit of 0.2% (0.04 ng DNA) was obtained in all meatball positive control, demonstrating the performance of our PCR assay under the complex background of meatball matrices. The Malaysian government has a strong commitment to build a halal hub in local and international domain and the Malaysian department of standards is responsible for halal authentication across Malaysia (Talib, Ali, Anuar, & Jamaludin, 2008). Therefore, our study was in line with the government policy and we found the validity and applicability of our assay for the detection of canine tissues both in raw and processed commercial halal products.

4. Conclusion
A short amplicon length (100 bp) cytochrome b based conventional PCR was documented for the detection of dog meat in commercial meatballs. The specificity test against 14 different species demonstrated that the assay was specific only for the dog cytb gene. Assessment under raw, ternary admixtures, meatball matrices along with cooking and extensive autoclaving condition, reflected that the method was suitable for the analysis of raw as well as processed meats. A blind experiment performed in triplicates on three different days by independent analysts, detected canine DNA only in positive controls. Screening of commercial meatballs from Malaysian supermarkets did not find any traces of canine tissues. The detection limit of the assay was 0.2% of dog meat (0.04 ng Dog DNA) from a complex DNA pool of meatball.

5. Ethical statement
Ethical clearance was obtained from Institutional Animal Care and Use Committee, University of Malaya (UM IACUC), Malaysia; (Reference number: NANOCAT/25/04/3013/MMR (R) and the meat samples were handled following the guideline for the proposed research.

Table 2
Meatball analysis using canine mt-cytb (100 bp) based PCR assay.

<table>
<thead>
<tr>
<th>Meatball sample</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Detection probability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure chicken meatball</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>0.9</td>
</tr>
<tr>
<td>Pure beef meatball</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>0.9</td>
</tr>
<tr>
<td>Pure dog meatball</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>0.9</td>
</tr>
<tr>
<td>Dog meat contaminated with chicken</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>27/27</td>
</tr>
<tr>
<td>Dog meat contaminated with beef</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>27/27</td>
</tr>
<tr>
<td>Commercial chicken meatball A</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>0.9</td>
</tr>
<tr>
<td>Commercial chicken meatball B</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>0.9</td>
</tr>
<tr>
<td>Commercial chicken meatball C</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>0.9</td>
</tr>
<tr>
<td>Commercial chicken meatball D</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>0.9</td>
</tr>
<tr>
<td>Commercial chicken meatball E</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>0.9</td>
</tr>
<tr>
<td>Commercial beef meatball A</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>0.9</td>
</tr>
<tr>
<td>Commercial beef meatball B</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>0.9</td>
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<tr>
<td>Commercial beef meatball C</td>
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<td>3</td>
<td>3</td>
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<tr>
<td>Commercial beef meatball D</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>0.9</td>
</tr>
<tr>
<td>Commercial beef meatball E</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>0.9</td>
</tr>
</tbody>
</table>
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